



Pathogens in model distribution system biofilms
by Malcolm Robert Warnecke

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:

Little is known about the behaviour of specific pathogenic microorganisms within a drinking water distribution system. This is particularly the case with surface-associated organisms, which have previously been demonstrated to have survival advantages over organisms in the bulk phase, and can serve as a reservoir of contamination. The drinking water environment can bring about physiological changes in organisms, leading to difficulties in their detection and changes in behaviour to that seen in other, more laboratory-oriented environments.

This study involved annular reactors operated to simulate a drinking water pipe environment. The reactors were initially seeded with a monoculture of one of two enteric pathogens (*Salmonella typhimurium* and *Escherichia coli* O157:H7) or two other bacterial strains of interest (*Aeromonas hydrophila* and *Klebsiella pneumoniae*). Following a period of establishment, a heterotrophic population was continuously added to the reactor. After the biofilm population was allowed to reach a pseudo-steady state, some reactors were treated with chlorine at levels typically observed in end-reaches of a distribution system. Samples of the bulk phase and the biofilm of the reactors were taken over the course of the experiment. These were examined using nonselective and selective media, and also by confocal microscopy using fluorescently labelled antibodies, DNA staining, and viability staining. Some colony identification was performed using the polymerase chain reaction.

The different bacteria studied showed significantly different behaviour under the tested conditions. *Salmonella typhimurium* rapidly became nondetectable with culture methods, but was still detectable using microscopy. It was found to persist in the biofilm, with at least some of these cells still viable. *Escherichia coli* O157:H7 did not persist well in the test reactors. *Aeromonas hydrophila* demonstrated variable behaviour between experiments, generally showing declining persistence and a loss of culturability in both bulk phase and biofilm, but an increase in culturable counts from growth or resuscitation in one replicate. *Klebsiella pneumoniae* showed a slow decline in culturability and overall numbers, rather than the growth expected with this organism. Chlorination reduced the numbers of test organisms in the reactors; cells in the biofilm were less affected by disinfection. Numbers of target cells determined by selective media were usually reduced to below detectable levels by chlorination, while immunofluorescence counts were less affected. Reactor conditions were shown to be similar between experiments by the levels of heterotrophic bacteria present.

Because the observed behaviour of similar bacterial pathogens was shown to be quite different in the test environment, it is unlikely that a single organism or group of organisms could be monitored to predict pathogen behaviour. Changes in bacterial physiology resulted in poor detection of pathogens using selective media. Surfaces can give pathogens various survival advantages, and are a likely reservoir for some organisms within a drinking water distribution system.

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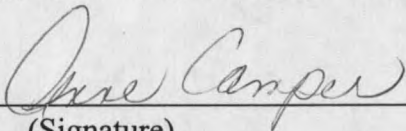
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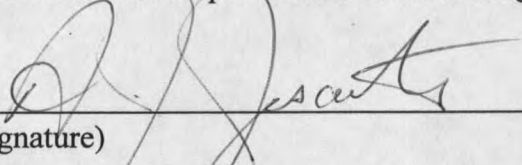
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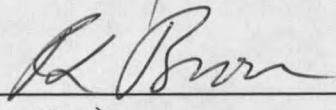
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TABLE OF CONTENTS

	Page
INTRODUCTION	1
Purpose of this study	1
Coliform testing	2
Physiological differences between coliforms and waterborne pathogens	3
Parasitic pathogens	3
Viral pathogens	4
Bacterial pathogens	5
Environmental stress responses within the bacterial cell	5
Injury	5
Starvation	7
Viable but nonculturable cells	8
Heterotrophs and opportunistic pathogens in distribution systems	9
The heterotrophic population	9
<i>Legionella</i>	11
Bacteria studied in this project	12
<i>Aeromonas hydrophila</i>	12
<i>Salmonella typhimurium</i>	15
A significant <i>S. typhimurium</i> outbreak in Gideon, Missouri ..	16
A major <i>S. typhimurium</i> outbreak in Riverside, California ...	16
<i>Escherichia coli</i>	17
A significant <i>E. coli</i> O157:H7 outbreak in Cabool, Missouri .	19
<i>Klebsiella pneumoniae</i>	20
The water distribution system as a microbial growth environment	21
Surfaces	22
Temperature	23
Corrosion products	24
Substrata	24
Nutrient levels	25
Indicator and pathogen detection and enumeration methods	26
The use of selective media	26
Alternative detection approaches to selective media	29
STATEMENT OF PROBLEM	34

TABLE OF CONTENTS - Continued

	Page
MATERIALS AND METHODS	36
Test organisms	36
Experimental system	36
Sampling protocol	40
Analytical methods	41
Culturing techniques	41
Microscopy techniques	42
Staining techniques	44
PCR techniques	46
Data analysis	47
RESULTS	49
Heterotroph data	49
Heterotroph variability between runs	49
Heterotroph behaviour in absence of disinfection	52
Heterotroph response to chlorination	56
Test organism data	58
<i>Salmonella typhimurium</i>	59
<i>Escherichia coli</i> O157:H7	65
<i>Aeromonas hydrophila</i>	70
<i>Klebsiella pneumoniae</i>	75
Disinfection effects upon test organisms	80
Examination of intact biofilms using microscopy	83
<i>Salmonella typhimurium</i>	83
<i>Escherichia coli</i> O157:H7	85
<i>Aeromonas hydrophila</i>	85
<i>Klebsiella pneumoniae</i>	88
Heterotrophic population	88
DISCUSSION	89
Use of coliforms to predict the behaviour of pathogens	89
Implications of observed physiological changes	90
Effects of surfaces	91
Behaviour of different organisms	92
Heterotrophic population	92

TABLE OF CONTENTS - Continued

	Page
<i>Salmonella typhimurium</i>	93
<i>Escherichia coli</i> O157:H7	94
<i>Aeromonas hydrophila</i>	95
<i>Klebsiella pneumoniae</i>	95
Utility of detection and identification methods	96
Selective media	96
Immunofluorescence	97
PCR confirmation	98
REFERENCES CITED	99
APPENDIX - RESULTS FROM EXPERIMENTAL RUNS	109

LIST OF FIGURES

Figure	Page
1. AOC solution component concentrations	37
2. Buffer solution component concentrations	38
3. Heterotrophs in unchlorinated bulk phase by 2day counts - linear regression.	50
4. Heterotrophs in unchlorinated bulk phase by 7day counts - linear regression.	50
5. Heterotrophs in unchlorinated biofilm by 2day counts - linear regression.	50
6. Heterotrophs in unchlorinated biofilm by 7day counts - linear regression.	50
7. Heterotrophs in unchlorinated bulk phase by PI counts - linear regression.	51
8. Heterotrophs in unchlorinated bulk phase by Rh123 counts - linear regression ...	51
9. Heterotrophs in unchlorinated biofilm by PI counts - linear regression.	51
10. Heterotrophs in unchlorinated biofilm by Rh123 counts - linear regression.	51
11. Mean unchlorinated bulk phase heterotroph values (2day R2A) over time	54
12. Mean unchlorinated bulk phase heterotroph values (7day R2A) over time	54
13. Mean unchlorinated bulk phase heterotroph values (PI) over time.	54
14. Mean unchlorinated bulk phase heterotroph values (Rh123) over time.	54
15. Mean unchlorinated biofilm heterotroph values (2day R2A) over time.	55
16. Mean unchlorinated biofilm heterotroph values (7day R2A) over time.	55
17. Mean unchlorinated biofilm heterotroph values (PI) over time	55
18. Mean unchlorinated biofilm heterotroph values (Rh123) over time.	55
19. Chlorination effects on heterotrophs in bulk phase.	57
20. Chlorination effects on heterotrophs in biofilm	57

LIST OF FIGURES - Continued

Figure	Page
21. Salmonella enumeration in bulk phase by XLD.	60
22. Salmonella enumeration in biofilm by XLD	61
23. Salmonella enumeration in bulk phase by Fab.	62
24. Salmonella enumeration in biofilm by Fab.	63
25. E. coli enumeration in bulk phase by mT7	66
26. E. coli enumeration in biofilm by mT7.	67
27. E. coli enumeration in bulk phase by Fab.	68
28. E. coli enumeration in biofilm by Fab	69
29. Aeromonas enumeration in bulk phase by selective media	71
30. Aeromonas enumeration in biofilm by selective media.	72
31. Aeromonas enumeration in bulk phase by Fab	73
32. Aeromonas enumeration in biofilm by Fab	74
33. Klebsiella enumeration in bulk phase by selective media	76
34. Klebsiella enumeration in biofilm by selective media	77
35. Klebsiella enumeration in bulk phase by Fab	78
36. Klebsiella enumeration in biofilm by Fab	79
37. Effects of 0.2ppm chlorine input on immunofluorescence counts of test organisms.	82
38. Unchlorinated Salmonella/heterotroph biofilm, week 2.	84
39. Unchlorinated Salmonella/heterotroph biofilm, week 6	84

LIST OF FIGURES - Continued

Figure	Page
40. Salmonella/heterotroph biofilm after 0.05ppm chlorination, week 3.	84
41. Salmonella/heterotroph biofilm after 0.2ppm chlorination, week 5.	84
42. Salmonella/heterotroph biofilm after nonselective enrichment..	86
43. Unchlorinated E. coli/heterotroph biofilm, week 1.	86
44. Unchlorinated Aeromonas/heterotroph biofilm, week 1.	86
45. Unchlorinated Aeromonas/heterotroph biofilm, week 2.	86
46. Aeromonas/heterotroph biofilm after 0.2ppm chlorination, week 4.	87
47. Unchlorinated Klebsiella/heterotroph biofilm, week 2..	87
48. Unchlorinated Klebsiella/heterotroph biofilm, week 2..	87
49. Klebsiella/heterotroph biofilm after 0.2ppm chlorination, week 4.	87
50. Sal Run 1 (Bulk Phase/Salmonella).	110
51. Sal Run 1 (Biofilm/Salmonella)	110
52. Sal Run 1 (Bulk Phase/Heterotrophs)	110
53. Sal Run 1 (Biofilm/Heterotrophs).	110
54. Sal Run 1C (Bulk Phase/Salmonella)	111
55. Sal Run 1C (Biofilm/Salmonella)	111
56. Sal Run 1C (Bulk Phase/Heterotrophs)	111
57. Sal Run 1C (Biofilm/Heterotrophs)	111
58. Sal Run 2 (Bulk Phase/Salmonella)	112
59. Sal Run 2 (Biofilm/Salmonella)	112

LIST OF FIGURES - Continued

Figure	Page
60. Sal Run 2 (Bulk Phase/Heterotrophs)	112
61. Sal Run 2 (Biofilm/Heterotrophs)	112
62. Sal Run 2C (Bulk Phase/Salmonella)	113
63. Sal Run 2C (Biofilm/Salmonella)	113
64. Sal Run 2C (Bulk Phase/Heterotrophs)	113
65. Sal Run 2C (Biofilm/Heterotrophs)	113
66. Sal Run 3 (Bulk Phase/Salmonella)	114
67. Sal Run 3 (Biofilm/Salmonella)	114
68. Sal Run 3 (Bulk Phase/Heterotrophs)	114
69. Sal Run 3 (Biofilm/Heterotrophs)	114
70. Eco Run 1 (Bulk Phase/Ecoli)	115
71. Eco Run 1 (Biofilm/Ecoli)	115
72. Eco Run 1 (Bulk Phase/Heterotrophs)	115
73. Eco Run 1 (Biofilm/Heterotrophs)	115
74. Eco Run 1C (Bulk Phase/Ecoli)	116
75. Eco Run 1C (Biofilm/Ecoli)	116
76. Eco Run 1C (Bulk Phase/Heterotrophs)	116
77. Eco Run 1C (Biofilm/Heterotrophs)	116
78. Eco Run 2 (Bulk Phase/Ecoli)	117
79. Eco Run 2 (Biofilm/Ecoli)	117

LIST OF FIGURES - Continued

Figure	Page
80. Eco Run 2 (Bulk Phase/Heterotrophs)	117
81. Eco Run 2 (Biofilm/Heterotrophs)	117
82. Eco Run 2C (Bulk Phase/Ecoli)	118
83. Eco Run 2C (Biofilm/Ecoli)	118
84. Eco Run 2C (Bulk Phase/Heterotrophs)	118
85. Eco Run 2C (Biofilm/Heterotrophs)	118
86. Aero Run 1 (Bulk Phase/Aeromonas)	119
87. Aero Run 1 (Biofilm/Aeromonas)	119
88. Aero Run 1 (Bulk Phase/Heterotrophs)	119
89. Aero Run 1 (Biofilm/Heterotrophs)	119
90. Aero Run 2 (Bulk Phase/Aeromonas)	120
91. Aero Run 2 (Biofilm/Aeromonas)	120
92. Aero Run 2 (Bulk Phase/Heterotrophs)	120
93. Aero Run 2 (Biofilm/Heterotrophs)	120
94. Aero Run 3 (Bulk Phase/Aeromonas)	121
95. Aero Run 3 (Biofilm/Aeromonas)	121
96. Aero Run 3 (Bulk Phase/Heterotrophs)	121
97. Aero Run 3 (Biofilm/Heterotrophs)	121
98. Kleb Run 1 (Bulk Phase/Klebsiella)	122
99. Kleb Run 1 (Biofilm/Klebsiella)	122

LIST OF FIGURES - Continued

Figure	Page
100. Kleb Run 1 (Bulk Phase/Heterotrophs)	122
101. Kleb Run 1 (Biofilm/Heterotrophs)	122
102. Kleb Run 1C (Bulk Phase/Klebsiella)	123
103. Kleb Run 1C (Biofilm/Klebsiella)	123
104. Kleb Run 1C (Bulk Phase/Heterotrophs)	123
105. Kleb Run 1C (Biofilm/Heterotrophs)	123
106. Kleb Run 2 (Bulk Phase/Klebsiella)	124
107. Kleb Run 2 (Biofilm/Klebsiella)	124
108. Kleb Run 2 (Bulk Phase/Heterotrophs)	124
109. Kleb Run 2 (Biofilm/Heterotrophs)	124

ABSTRACT

Little is known about the behaviour of specific pathogenic microorganisms within a drinking water distribution system. This is particularly the case with surface-associated organisms, which have previously been demonstrated to have survival advantages over organisms in the bulk phase, and can serve as a reservoir of contamination. The drinking water environment can bring about physiological changes in organisms, leading to difficulties in their detection and changes in behaviour to that seen in other, more laboratory-oriented environments.

This study involved annular reactors operated to simulate a drinking water pipe environment. The reactors were initially seeded with a monoculture of one of two enteric pathogens (*Salmonella typhimurium* and *Escherichia coli* O157:H7) or two other bacterial strains of interest (*Aeromonas hydrophila* and *Klebsiella pneumoniae*).

Following a period of establishment, a heterotrophic population was continuously added to the reactor. After the biofilm population was allowed to reach a pseudo-steady state, some reactors were treated with chlorine at levels typically observed in end-reaches of a distribution system. Samples of the bulk phase and the biofilm of the reactors were taken over the course of the experiment. These were examined using nonselective and selective media, and also by confocal microscopy using fluorescently labelled antibodies, DNA staining, and viability staining. Some colony identification was performed using the polymerase chain reaction.

The different bacteria studied showed significantly different behaviour under the tested conditions. *Salmonella typhimurium* rapidly became nondetectable with culture methods, but was still detectable using microscopy. It was found to persist in the biofilm, with at least some of these cells still viable. *Escherichia coli* O157:H7 did not persist well in the test reactors. *Aeromonas hydrophila* demonstrated variable behaviour between experiments, generally showing declining persistence and a loss of culturability in both bulk phase and biofilm, but an increase in culturable counts from growth or resuscitation in one replicate. *Klebsiella pneumoniae* showed a slow decline in culturability and overall numbers, rather than the growth expected with this organism. Chlorination reduced the numbers of test organisms in the reactors; cells in the biofilm were less affected by disinfection. Numbers of target cells determined by selective media were usually reduced to below detectable levels by chlorination, while immunofluorescence counts were less affected. Reactor conditions were shown to be similar between experiments by the levels of heterotrophic bacteria present.

Because the observed behaviour of similar bacterial pathogens was shown to be quite different in the test environment, it is unlikely that a single organism or group of organisms could be monitored to predict pathogen behaviour. Changes in bacterial physiology resulted in poor detection of pathogens using selective media. Surfaces can give pathogens various survival advantages, and are a likely reservoir for some organisms within a drinking water distribution system.

INTRODUCTION

Purpose of this study

A drinking water distribution system is a complex heterogeneous environment, potentially serving as both a conduit and a growth site for pathogenic bacteria. These organisms have frequently been well characterized in the clinical microbiological setting, but their behaviour can change in the environment of drinking water systems. This can lead to various difficulties in recognition of an organism as a public health threat, as well as its detection and control in the distribution system environment. The overall aim of this project is to determine what behaviour can be expected of selected bacteria of concern within this system, with regard to their persistence, survival, physiological changes, and interactions with other organisms. Of particular interest is the ability of cells to persist in or form biofilms, and to then subsequently detach into the bulk phase of distributed water.

There are many considerations influencing the behaviour of microorganisms within a distribution system. This introduction aims to briefly summarize the current understanding of various factors influencing pathogens in the drinking water environment, such as surfaces, nutrient levels and disinfection. Particular emphasis is given to the various bacterial pathogens studied in this project, and their relevance in waterborne disease and behaviour in distribution systems. Various detection methodologies under recent development are also briefly discussed, and compared with the more standardized methods currently in common usage.

Coliform testing

Since early in the 20th century, coliform enumeration has been the primary method of monitoring water quality (Pipes, 1990). Coliforms are defined as aerobic and facultative gram-negative nonspore-forming rod-shaped bacteria which ferment lactose with gas and acid production at 35°C within 48 hours (APHA, 1992). In practical terms this includes several members of the bacterial family *Enterobacteriaceae*. As these organisms are normal flora in the enteric tract of warm-blooded animals, water containing them may have been contaminated with fecal material, including pathogenic organisms.

The intended significance of coliforms in water is therefore as indicators of fecal pollution. There are many pathogenic organisms with demonstrated fecal-oral spread and potential for waterborne transmission. To verify water supplies as free of them all would be impractical, and in some cases the task would be impossible. This is due to the sheer number of pathogens that would have to be tested for, usually with expensive and time-consuming methods that are frequently unreliable and require very large sample volumes. For example, the standard method for *Cryptosporidia* detection in water requires experienced technicians, over 100 liters of water sample volume, and frequently results in recovery of less than 5% (Vesey et al, 1993a). Using the coliform test, and the fecal or thermotolerant coliform test (fermentation of lactose with gas production at 44.5°C within 24 hours - APHA, 1992) to indicate fecally compromised water has been of much benefit to public health. The major waterborne disease threats at the turn of the century, such as

typhoid fever and cholera, have been largely controlled in developed countries by use of coliform monitoring and chlorine disinfection.

Coliforms are thus well accepted as potential indicators for recent fecal pollution in drinking water supplies, and of the efficiency of water treatment processes in removing this pollution. However, care must be taken when interpreting the indicative value of such data. To predict the behaviour of different pathogens under a wide variety of circumstances, indicator organisms would have to mimic the pathogen's physiology almost perfectly. These circumstances would include, for example, potential for regrowth in the system, tolerance to chlorine-induced injury, and ability to survive for extended periods in the drinking water environment. It seems likely the predictive role of indicators could be limited in these applications.

Physiological differences between coliforms and waterborne pathogens

Parasitic pathogens

The most obvious differences between some types of fecally-derived waterborne pathogens and coliforms is on a kingdom level, specifically with viruses and protozoa. A recent example of the importance of this was the failure of coliform testing to indicate a waterborne outbreak of approximately 403,000 cases in Milwaukee, Wisconsin, with the etiologic agent being the protozoan *Cryptosporidia* (MacKenzie et al, 1994). This organism has the capacity to exist as an oocyst, giving it long term persistence and chlorine resistance. The protozoan *Giardia* has demonstrated similar chlorine resistance in

its cyst form; this is emerging as the leading identified cause of waterborne disease in the USA today (Craun, 1986; Craun et al 1991; Herwaldt et al 1992; Moore et al 1994).

These organisms have long-term environmental persistence, show some association with turbidity, and usually are not well correlated with coliform levels. They can demonstrably pass disinfection barriers in the distribution system, and their behaviour once in the system is largely unknown. Some initial studies have indicated oocysts adhere very well to preexisting biofilm (Rogers et al, 1996). Hence, the subsequent detachment of biofilm containing large numbers of oocysts could explain infectivity and difficulties in the detection of these organisms in water. *Entamoeba histolytica* is an infrequent but still endemic waterborne pathogen in the USA today. It infects the gastrointestinal tract, frequently asymptotically (Craun, 1986).

Viral pathogens

Over 120 different viruses can be excreted in feces and urine, and become water pollutants leading to a wide spectrum of diseases (Rao and Melnick, 1986; Kapikian et al, 1996). This includes such groups as the picornaviruses including enteroviruses and hepatitis A virus, caliciviruses including the Norwalk agent, and others such as rotaviruses. Agents such as the hepatitis A virus have demonstrated extended survival in the aquatic environment (Gerba and Rose, 1990). The prevalence and ability of viruses to persist in water distribution systems is generally unknown, along with the effects their survival has upon the community. However, viruses were identified as the cause in 12% of waterborne outbreaks in the USA between 1946 and 1980 (Gerba and Rose, 1990), and that given the

difficulties in isolation this figure is undoubtedly under-reported.

Bacterial pathogens

Bacterial enteric pathogens identified from waterborne outbreaks include *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, enterotoxigenic and enterohemorrhagic *Escherichia coli* (ETEC and EHEC), *Yersinia enterocolitica*, along with *Vibrio* spp. and *Salmonella typhi* in developing countries (Craun, 1986). Survival of these organisms varies greatly, ranging from the poor survival of *Campylobacter* to an estimated survival of *Y. enterocolitica* in well water of 540 days (Highsmith et al, 1977).

Environmental stress responses within the bacterial cell

Injury

The importance of and processes related to injury from various sources in coliforms have been summarized by McFeters (1990). Because indicator organisms and pathogenic bacteria are not well adapted to conditions in water, they can become physiologically damaged with aquatic exposure. This results in an inability to reproduce under selective or restrictive conditions tolerable by uninjured cells. Highly selective media, such as those usually employed to enumerate coliforms and fecal coliforms, will therefore incorrectly estimate numbers of target cells, as it will restrict growth of cells injured past a certain level. However, this cellular injury is reversible, and debilitated cells can be resuscitated under appropriate conditions. In drinking water, disinfection is

probably the most significant cause of injury, particularly at sub-optimal (non-lethal) levels. Metal ions, UV radiation, acid conditions, and biological interactions are also noted as potentially injurious stresses, causing damage primarily to the cell membrane. Studies have shown the levels of injury to be high in distribution systems, and highly selective methods may be underestimating coliforms by orders of magnitude. The overall significance of this is the potential generation of false-negative coliform results from water testing, obscuring the coliform's usefulness to signal health hazards.

The effects of injury upon pathogenic bacteria, particularly when related to coliforms, are also of significance, and have been summarized by Singh and McFeters (1990). The primary question is whether injured pathogens retain their ability to cause disease. There may be a temporary loss or reduction of virulence, caused by damage to surface components responsible for attachment or invasiveness. Damage can be repaired under suitable conditions, such as enrichment media or the small intestine of a host; and thus the health hazard of the organism may be undiminished. Other consequences are also possible, including altered physiology towards dormancy. The behaviour of different bacterial pathogens under stressful conditions varies widely, and is influenced by other factors such as cell concentration, contact time, and temperature. Pathogens such as *Y. enterocolitica*, *S. typhimurium*, and *Shigella* sp. were found to be more chlorine resistant than coliform bacteria, whereas *Campylobacter jejuni* and enterotoxigenic *E. coli* (ETEC) were found to be inactivated and injured at lower chlorine doses.

Starvation

A similar phenomenon is that of reduced culturability and therefore detectability of organisms taken from a low-nutrient or "oligotrophic" environment. This appears to be an active reaction to physiological stress by many bacteria, taking the form of the "starvation" response, or an apparently related response leading to "viable but nonculturable" (VBNC) cells.

Many waterborne pathogenic bacteria are adapted to growth at high nutrient levels. When confronted with nutrient limitation they have been shown to undergo certain physiological and morphological changes, collectively known as the starvation response (Matin et al, 1989). This response is under global genetic control in the cell. Some observed responses have included macromolecular degradation of cellular RNA and protein, a decrease in size and increases in adhesive properties to take advantage of surface-congregating nutrients (Marshall, 1988; Matin et al, 1989). These changes appear dependant upon an intense period of metabolic activity at the start of the response (Morita, 1988), and are reversible following nutrient addition to the cells (Morita, 1988; Marshall, 1988), although with a lag time proportional to the period of starvation.

Of major significance in drinking water distribution systems is the observation that cells undergoing the starvation response are markedly more disinfectant resistant than rapidly growing cells (Matin and Harakeh, 1990). It is likely that this resistance is conferred by the production of stress proteins in starved cells which help protect them against a variety of environmental stresses. The amount of resistance is generally determined by the extent of nutrient limitation, and other factors such as temperature. The

longer the cell has been starved, the greater the disinfection resistance conferred. This has been observed in several organisms of concern to the drinking water industry, such as *Y. enterocolitica*, *E. coli*, and *L. pneumophila*. The exact mechanism of protection by stress proteins is not known, although it is thought to involve factors such as the regulation of metabolism in the cell, and nutrient uptake affinity mechanisms.

Viable but nonculturable cells

A related phenomenon is the formation of viable but nonculturable, or VBNC cells, recently extensively reviewed by Oliver (1993). This differs from injury in that cell behaviour is related to starvation, and results in reduced culturability on all media rather than just selective media. Affected cells remain metabolically active, and can "resuscitate" to a culturable state given appropriate conditions such as human passage. VBNC studies have examined predominantly marine *Vibrio* spp., although this condition has been observed in many of the gram-negative pathogen and indicator bacteria. It appears to be triggered by stresses such as temperature shifts rather than xenobiotic compounds, although the inducing environmental conditions vary widely depending on the bacterium. It differs from the starvation response in that resuscitation of VBNC cells can take hours or days rather than a rapid reversal of minutes to hours. However, there appears to be some relationship between these conditions, with starved cells demonstrating a slower entry into the VBNC state than log-phase cells, following appropriate triggering conditions for inducing VBNC cells.

The significance of the VBNC condition is much the same as that of injured cells,

with the potential for indicators and pathogens to be undetected by commonly used culture media.

Heterotrophs and opportunistic pathogens in distribution systems

The heterotrophic population

Distribution system waters are known to have the potential for containing a wide variety of heterotrophic organisms in large numbers. These organisms are routinely enumerated by water utilities using broad spectrum, low nutrient growth media such as R2A agar (Reasoner and Geldreich, 1985), or by total count methods. Little is known of the gastrointestinal pathogenic potential of this heterotrophic population.

Infections caused by general heterotrophs may be quite specific and generally unnoticed. For example, they could cause sub-clinical symptoms, or only affect vulnerable parts of the consumer population such as the immunocompromised. Bodily areas other than the gastrointestinal tract may also be the site of infection such as the ear, nose or throat (Reasoner, 1991), or the respiratory tract in the case of Legionnaires Disease (see below). In the bulk of recent waterborne outbreaks of gastroenteritis in the USA, the etiologic agent has remained unidentified (Craun et al, 1991; Herwaldt et al 1992; Moore et al 1994), often along with the absence of coliform indicator bacteria using conventional media. These infections could be caused by unrecognized pathogens amongst the heterotrophic population, as well as viral or protozoan pathogens.

It has been shown (Payment, 1991) that consumers of distribution system water in a metropolitan system had a greater risk of low-level gastrointestinal illness than a control group consuming the same water after reverse osmosis filtration. This was manifested as an increase in number of episodes of gastrointestinal illness amongst susceptible individuals, rather than increasing the number of people with illness. The data from this study were further analysed (Payment, 1993), and while there was no correlation of illness with coliform levels, there was association with heterotrophic plate counts incubated at 35°C, and with increasing distance from the water treatment plant. This correlation with distance was attributed to regrowth of bacteria, as sewage cross-connections would have resulted in an epidemic distribution of cases, and viruses or parasites would have been further inactivated with increased exposure time in the distribution system. From this epidemiological study, it could be concluded that the enumeration of heterotrophs should be included with coliform monitoring of water supplies. It has been further suggested (Payment et al, 1994) that assaying the virulence of heterotrophic bacteria may be of more practical use than merely assessing their numbers.

This potential for low-level endemic infection is becoming increasingly important particularly with a growing immunocompromised proportion of the community, as a result of aging and spread of the HIV virus. There remains the possibility that organisms thought to be benign in a distribution system might be the etiologic agent in waterborne outbreaks of disease. For example, both *Klebsiella pneumoniae* and *Aeromonas hydrophila* may be isolated from well-maintained systems, and be considered harmless. However, both of these organisms are capable of expressing virulence factors, and are the causative agents

for other, non-waterborne human infections (see the later section on these bacteria).

In addition to potentially containing opportunistic pathogens, the heterotrophic population can also be a considerable nuisance. Heterotrophs can cause false positives to the coliform test, mask coliforms by overcrowding on filter membranes (APHA, 1992), or generate taste and odor problems (O'Connor et al, 1975; Servais et al, 1993).

Legionella

An atypical opportunistic pathogen which has acquired some notoriety in recent years is *Legionella pneumophila*. This organism produces an opportunistic respiratory disease (Legionnaire's Disease) as opposed to a gastrointestinal one, and is spread by aerosolized infective particles, originating from sources such as air conditioning cooling towers and shower heads (Edelstein and Meyer, 1984). The organisms itself is quite fastidious with regards to growth requirements, but is widespread in its natural aqueous environment. It is not readily isolated from drinking water, although in some cases drinking water is able to support growth of added *Legionella* following chlorine neutralization (States et al, 1987). *Legionella* incidence is complicated by its niche as an intracellular pathogen of free-living protozoa, which themselves graze upon bacteria usually congregated in the biofilm of a distribution system. The infection of protozoa can lead to production of vesicles laden with legionellae which serve as infective particles, or protozoan cysts containing legionellae which are highly resistant to disinfection (Rowbotham, 1986). *Legionella* is a temperature dependant genus that can cross-feed with other microbial populations to meet its growth requirements (Tison et al, 1980;

Rogers et al, 1994a and 1994b); this type of community interaction is likely only in the biofilm of a water distribution system. Events which detach biofilm, such as maintenance of household hot water systems, have potential to release large numbers of *Legionella* into the water phase. Detachment as a source of infection appears to be frequently unrecognized. Epidemiologic efforts to isolate the mode of exposure frequently end inconclusively (Fraser, 1985), presumably due to lack of biofilm sampling.

Bacteria studied in this project

Aeromonas hydrophila

Aeromonads in drinking water systems have been the subject of much scrutiny and conjecture in recent years. They are considered water-based rather than water-borne organisms, since they are indigenous to aquatic environments and physiologically adapted for growth within the drinking water system (Rippey et al, 1979). Aeromonads appear to be a small and variable component of the overall heterotrophic population (Havelaar et al, 1990), with conflicting evidence sometimes supporting the correlation of aeromonads with the overall heterotrophic plate count population (Havelaar et al, 1990; LeChevallier, 1982). They can use a wide range of biopolymers; this may be important for aeromonads to maintain viability or grow in a distribution system, particularly if the aeromonads are growing in the biofilm using products from more predominant bacteria (Van der Kooij, 1988). It has been shown that the presence of other microflora such as pseudomonads in bottled water enhanced the survival of *Aeromonas* (Warburton, 1993).

The factors governing aeromonad regrowth appear highly complex and are not well understood. In some studies, positive correlations have been made with temperature and residence time (Havelaar et al, 1990), although temperature has been shown to have no correlation in other investigations (Burke et al, 1984b). However, these studies collectively agree that aeromonad numbers have no correlation to coliform levels.

Aeromonad taxonomy is also highly confusing. Based on phenotype and DNA-DNA reassociation kinetics (Janda, 1991), three species are generally accepted as most clinically important (*Aeromonas hydrophila*, *A. caviae*, and *A. sobria*). The different species have been typically associated with a wide range of opportunistic skin and soft tissue infections. The source is usually water or soil (Khardori and Fainstein, 1988; Altwegg and Geiss, 1989). All three species have been found in potable water distribution systems, and isolated from stools of patients with diarrhea. *A. caviae* the most commonly isolated aeromonad from such patients (Van der Kooij, 1988).

The potential for waterborne aeromonads to act as etiological agents in a disease outbreak is also poorly understood. It has been shown (Burke et al, 1984a and 1984b) that aeromonad numbers in the distribution system correlated with the numbers of clinical gastroenteritis cases associated with aeromonads. In a survey of studies investigating the presence and absence of aeromonads in feces, particularly of diarrhea patients (Van der Kooij, 1988), it was found that isolation rates varied widely (<1% to 20%), with highest isolation frequencies in tropical regions and lowest in Europe and the USA. The most common isolate was *A. caviae*. He also notes that the isolation of aeromonads in the absence of other pathogens is not adequate evidence of it being the etiologic agent.

Virulence factors in aeromonads have been demonstrated by many studies (LeChevallier et al, 1982; Burke et al, 1984a; Altwegg and Geiss, 1989; Janda, 1991), and include adherence factors, various hemolysins, cytotoxins, enterotoxins and proteases. Together with data from other varied aeromonad infections, these organisms can clearly be pathogenic. When isolated from drinking water systems, they often possess these virulence factors (above studies, also Gray et al 1990). However, it has been observed (Van der Kooij, 1988) that despite the widespread occurrence of aeromonads in drinking water, epidemic outbreaks of *Aeromonas*-caused diarrhea have not been reported. There has also been a noted lack of correlation between the known virulence factors in aeromonads and human pathogenicity (Morgan et al, 1985).

Concerns associated with aeromonads have led to some attempts at regulation of these organisms. For example, health authorities in the Netherlands have defined 20 CFU/100mL in drinking water at the production plant and 200 CFU/100mL during distribution as maximum allowable values (Van der Kooij, 1988). In Canada, a limit of 0 CFU/100mL in bottled water has been proposed (Warburton et al, 1994).

Overall, it can be concluded that while *Aeromonas* demonstrably has a niche in distribution system microbiota and possesses the potential for causing waterborne disease in humans, it is unclear as to exactly what role it plays in both of these fields of interest.

Salmonella typhimurium

Salmonella is well recognized as an organism of fecal origin. It therefore should not be present in a well-run potable water distribution system. If found in water, it is regarded as more a case of survival rather than proliferation for the organism in such conditions. This survival possibility has been demonstrated in the outbreaks at Riverside, California, and at Gideon, Missouri, as described below.

The importance of attachment to environmental surfaces in this survival phenomenon has been previously investigated. Camper et al (1985) found *Salmonella* readily colonized and persisted on granular activated carbon in water, although attachment was at a lower rate and the organism decreased in numbers more rapidly in the presence of other heterotrophic bacteria. Suspended pathogen cells died away faster than attached cells. Longer term survival of attached *Salmonella* in water conditions appears to be a common observation from this study.

With the exception of some very large outbreaks such as Riverside, there seems to be a level of about 1-100 cases per year of waterborne salmonellosis (Craun, 1986). There is considerable potential for non-detection, given phenomena such as viable non culturable (VBNC) cells with *Salmonella*. In an investigation with *Salmonella enteritidis* in river water microcosms (Roszak et al, 1983), salmonellae rapidly became nonculturable. This was initially reversible following nutrient addition, but after 3 weeks resuscitation failed to give culturable cells, although the cells remained viable. Therefore, even if water during a disease outbreak tests negative for salmonellae, there remains the possibility of transmission by VBNC cells.

A significant *S. typhimurium* outbreak in Gideon, Missouri was reported by Clark et al, 1996. This waterborne outbreak was attributed to a large municipal storage tank in poor repair, with bird's feathers and droppings present in the tank. A temperature inversion led to the mixing of tank sediments with the water column, resulting in consumer taste and odor complaints. This led to a city-wide flushing of the supply system with the contaminated stagnant tank water.

Tank sediments were found to contain *Salmonella* of the same serovar as outbreak patients. Water samples taken through the outbreak were generally negative for coliform and fecal coliform detection, although some were positive at low levels. The *Salmonella* strain was shown to survive well in the town water, demonstrating only a 30% drop in numbers after 4 days at 15°C.

There seems a good case here for a long-term buildup of sediment-associated salmonellae, spread by a sudden release of the resuspended sediment. The uncertain correlation of indicator bacteria with the salmonellae in this case could be due to differing survival potentials of these organisms in this environment. If numbers of contaminating indicators had dropped sufficiently over time, they could not indicate the presence of surviving salmonellae.

A major *S. typhimurium* outbreak in Riverside, California is described by Boring et al, 1971, and a collaborative report, 1971. This outbreak was traced to a single well in the town supply system, although no contamination event was established. Prior to and during the outbreak this well supplied the town system intermittently, with several periods of 12-

48 hours when the pumps were not working.

Coliform test results before and during the outbreak revealed no significant contamination. *Salmonella* of the same type as the outbreak cases was isolated from the water supply in the vicinity of the suspect well. It was estimated that the *Salmonella* source was present for about 12 days and then disappeared when chlorination was begun, although the two events were not necessarily linked.

The limited *Salmonella* quantification performed revealed only low numbers (10^1 - 10^3 cells per liter), whereas food-borne *Salmonella* usually requires a large infective dose ($\sim 10^6$ cells). This apparent discrepancy was postulated as being possible, as water has a shorter residence time in the stomach than foodborne *Salmonella* infections, thus requiring a lower infective dose.

It could be speculated that this outbreak was caused by sediment- or biofilm-associated salmonellae being released into or within the distribution system. Resuspension of the bacteria could have been caused by the recorded changes in hydraulics, or some other environmental change. The apparently low infective dose could also be explained if large numbers of organisms were associated with particles or a released "bolus" of biofilm, resulting in a highly uneven distribution through the water phase, producing large numbers of cells in single infectious particles which were protected from stomach acids.

Escherichia coli

The standardized use of the coliform and thermotolerant coliform enumeration indicator tests to assess microbial water quality has stimulated many studies on the

behaviour of *E. coli* in water systems, as it can make up a significant proportion of coliforms, and almost all of the thermotolerant coliforms. Its use as an indicator assumes that *E. coli* originates exclusively from fecal contamination, and survives in a fashion approximating that of fecal pathogens. The former assumption is becoming increasingly challenged from results in warm climatic areas of the world, where environmental isolates of *E. coli*, perhaps associated with the phyllosphere, are commonly isolated from water. This has occurred in dune systems near Rio de Janeiro, Brazil (Hagler et al, 1993), source waters in Puerto Rico (Rivera et al, 1988), and water storages in Sydney, Australia (personal observations, unpublished). The use of *E. coli* as an indicator of fecal pollution is thus of less use in these systems.

Some strains of *E. coli* such as the enterohemorrhagic O157:H7 strain (ECO157) have a demonstrated pathogenicity to humans. This strain has several physiological differences from typical isolates, including a lack of the enzyme β -glucuronidase (often used for *E. coli* detection) and poor or no growth at 45°C (Rice et al, 1992). Therefore, this strain is non-detectable by standard methods used for the routine detection of *E. coli*. It has been shown to persist at a similar rate as typical *E. coli* strains under drinking water conditions, confirming the premise that typical *E. coli* strains would be effective in indicating the ECO157 presence (Rice et al, 1992). However, this study did not account for differential survival on particulates or in biofilms. Survival on surfaces has been shown to be the major long-term persistence mechanism of *E. coli* in lake waters (Brettar and Höfle, 1992). Pathogenic and non-pathogenic *E. coli* strains have been shown to have similar growth rates to an environmental isolate of *E. coli* under growth conditions

relevant to drinking water distribution systems (Camper et al, 1991).

Colonization by an environmental *E. coli* isolate of a preexisting biofilm has been demonstrated under water distribution system conditions (Robinson et al, 1995). A fecal origin, non-benzoate degrading *E. coli* has been shown to be able to colonize a reactor containing a biofilm of benzoate degrading bacteria, and subsequently re-enter the water phase. In this study, 5mM benzoate was the sole carbon source, demonstrating consortial feeding by this organism (Szewzyk et al, 1994).

A significant *Escherichia coli* O157:H7 outbreak in Cabool, Missouri was reported by Geldreich et al, 1992; and Swerdlow et al, 1993. Prior to this outbreak, sections of a water main in Cabool were replaced followed by flushing but without hyperchlorination. This area was also in the vicinity of various sewage overflows. A single event of system contamination by backflow during repairs was concluded as the likely source of the outbreak. ECO157 was not isolated from the water supply, although this is not unusual for a waterborne outbreak.

Cases of ECO157 infections decreased after a boil order was issued and chlorination of the distribution system began. Towards the end of the outbreak the incidence of bloody as opposed to non-bloody diarrhea amongst case patients decreased, which was attributed to ingestion of lower doses of ECO157. It was estimated that ECO157 survived up to 2 weeks in the distribution system.

From this outbreak report it may be concluded that the possibility exists that there was a single large inoculum of ECO157 into the distribution system. This contamination

was then disseminated through and flushed out of the system by routine water usage. It may have not persisted in the system, as seen by the change of patient symptoms resulting from diminishing doses of infection.

Klebsiella pneumoniae

This organism has caused some concern in the drinking water industry, due to its demonstrated capacity to colonize and regrow in distribution systems and also by causing a positive coliform test (Geldreich and Rice, 1987; unpublished data). This suggests the possibility that a bloom of *Klebsiella* could generate a false indication of fecal contamination within the system that could potentially mask a true coliform positive result. Most *Klebsiella* species have been detected from water systems by the coliform test, although some *K. pneumoniae* elicit a positive result with the fecal coliform test as well. The latter organisms can be regarded as of either fecal or environmental origin, as this species is associated with the gastrointestinal tract of many warm-blooded animals and also with plant products (Geldreich and Rice, 1987; Geldreich, 1991a and 1991b).

The public health significance of environmental *Klebsiella* is not clear. Using mouse infectivity models, *Klebsiella* from diverse environmental origins, regardless of fecal coliform response or biotype, have been demonstrated to be potentially as pathogenic as isolates of clinical origin (Bagley and Seidler, 1978). This contrasted with tested *E. coli* and *Salmonella* isolates, which demonstrated diminished virulence upon entry into the environment. The presence of multiple antibiotic-resistant *Klebsiella* in water would be of potential concern for susceptible consumers (Geldreich and Rice, 1987); although it would

appear that these strains are found in nosocomial infections rather than acquired in the community (Smith et al, 1982). It has been observed that klebsiellae infections are seen mainly in the hospital and are rare in the community; this is attributed to the colonization of susceptible patients rather than the hospital environment itself (Montgomerie, 1979). It is possible that a susceptible population within the community could be vulnerable to waterborne exposure to this opportunistic organism.

There is a lack of evidence of increased illness in a community during a biofilm regrowth event of klebsiellae (Geldreich, 1991b), potentially due to difficulties in gathering reports of water related illness amongst susceptible individuals in the community. The epidemiological difficulties of linking generalized opportunistic infections (gastrointestinal, respiratory, urinary, wound) with such an event would be immense, and probably render such an exercise impractical.

The water distribution system as a microbial growth environment

An environment containing low levels of nutrients and often biocides at levels designed to kill microorganisms would not seem like a good growth environment. However, the universal presence of heterotrophic bacteria in distribution systems would indicate that growth is very possible in these systems, or that organisms from source waters are persisting through the system.

Surfaces

Interaction with surfaces in these systems is one of the more important growth determinants. In pilot plant studies it has been shown that growth in the bulk liquid phase is negligible and that planktonic increases are due primarily to detachment of biofilm cells and "breakthrough" of cells through the water treatment plant barriers (Van der Wende et al, 1989; Bucklin et al, 1991; Block, 1992). A biofilm has been defined as a surface accumulation of cells immobilized at a substratum, frequently embedded in an organic polymer matrix of microbial origin, which may contain a significant fraction of inorganic or abiotic substances (Characklis and Marshall, 1990). Bacterial attachment to suspended particles or to the pipe wall can give substantial advantages to these cells. As summarized by Fletcher and Marshall (1982), (1) substrata-attached cells do not have to waste energy searching for food, as water containing fresh nutrients flows over them and removes waste products at the same time; (2) they are prevented from washing through the system; (3) the substrata attached to may also be a growth substrate; and (4) nutrients tend to adsorb to surfaces and are thus more available to surface attached cells.

The protective effect of attachment from disinfection is also very significant. Attachment led to the single greatest increase in disinfection resistance in one study of several potential factors (LeChevallier et al, 1988). Disinfectant-resistant cells can potentially be transported through water treatment barriers and throughout the distribution system by suspended particles (LeChevallier et al, 1984). It was demonstrated in this study that cells colonized cracks and crevices of granular activated carbon grains, produced extracellular polymeric substances, and were effectively resistant to 2.0 mg/L of chlorine.

These findings agree with Herson, et al (1987), where it was found that cells readily colonized suspended particles, and that attachment gave enhanced chlorine resistance.

With the increasing usage of carbon to filter organics from source waters, the opportunity for pathogen breakthrough into the water system on carbon particles is also increased.

Attachment to pipe surfaces can give protection in the same fashion as attachment to particles. An examination of distribution system biofilms found no correlation between biofilm heterotrophic plate counts and the bulk phase free chlorine residual (Nagy and Olson, 1985). A pilot system study (Van der Wende et al, 1989), found that chlorine influenced biofilm location within a system, with it tending to form later in the system once the chlorine residual was lower.

Temperature

Water temperature is usually correlated with heterotrophic growth rates. It is attributed as a major factor in seasonal regrowth events, although there is the possibility of other seasonal effects such as increased nutrient levels affecting water quality. Donlan and Pipes (1988) found that the attached microbial population density was directly related to water temperature. LeChevallier et al (1991) found coliform regrowth increased at temperatures over 15°C. Camper and Jones (1996) found growth rates of heterotrophs higher at 20°C than 10°C under distribution system conditions. This could be expected due to increased cellular enzyme reaction rates at the higher temperatures.

Corrosion products

The role of corrosion products in the population dynamics of bacteria in potable water distribution systems is not entirely clear. In cast-iron mains, it is contended that microbial induced corrosion is largely responsible for rusting (Victoreen, 1984a). The resultant corrosion formations or "tubercles" have been found to harbour large numbers of microorganisms including coliforms. Pulverized tubercle material, largely consisting of rust, has been found to enhance growth of heterotrophs and coliforms in some studies (Allen and Geldreich, 1977; Victoreen, 1984b) but not in others (Camper et al, 1991). This could be explained by the rust acting as a biological catalyst (Victoreen, 1984b) or as nutrient source, dependant upon the chemistry of the water and substratum, and the microflora present. The protective effect of tubercles from predation and disinfection could also explain their preferential colonization.

Substrata

The original pipe surface properties greatly influence the subsequent growth upon it, and hence the water quality within the pipe. Comparative testing of pipe surfaces under drinking water conditions to assess biofilm development has been performed in several studies (Block, 1992; Rogers et al, 1994a and 1994b; Camper and Jones, 1996). The results of these studies indicate that the smoother and less corrodable surfaces are less attractive for bacterial colonization. Leaching from the substrata of potentially inhibitory ions or of organic nutrients also affects colonization (Rogers, 1994a and 1994b).

Nutrient levels

Nutrient levels in the water itself are an obvious determinant of microbial growth. For example, there has been an association of the level of assimilable organic carbon (AOC) with microbial growth in some studies. LeChevallier et al (1991) found that an AOC level of greater than $50\mu\text{g L}^{-1}$ of acetate-carbon equivalents correlated with the occurrence of coliforms in one distribution system, and concluded AOC levels should be kept below this concentration to limit coliform regrowth. AOC was the only nutrient parameter observed to decline as water moved through the distribution system, and AOC levels also correlated with heterotroph numbers. Van der Kooij (1992) found a significant correlation between concentration of AOC leaving the water treatment plant and heterotroph counts within the water phase, suggesting that AOC uptake by biofilm cells is potentially reflected by the number of suspended cells. He concluded that AOC concentrations of less than $10\mu\text{g L}^{-1}$ acetate-carbon equivalents could be used to limit bacterial growth potential. Camper and Jones (1996), in a pilot plant study, found the number of organisms to be elevated at higher substrate levels, but found that the overall heterotrophic growth rate was not affected by carbon substrate concentration.

“Typical” AOC levels in water systems are difficult to determine. A recent single-sampling survey of large participating US utilities found 18-322 $\mu\text{g L}^{-1}$ of C equivalents in water supplies (Kaplan et al, 1994). A study of a particular system over a period of time by Huck, et al (1991) found much seasonal variability, with raw water AOC values peaking as high as $610\mu\text{g L}^{-1}$ during early spring and usually lower during the rest of the year. It was suggested that waters having different temperature regimes, concentrations

of, or sources of organic matter would influence AOC levels and regrowth. An example would be precipitation causing runoff into source water resulting in the introduction of nutrients (LeChevallier et al, 1991).

There is a need for using AOC as a growth determinant parameter, as opposed to simpler and more easily determinable parameters such as dissolved organic carbon (DOC). DOC levels have no correlation with heterotroph numbers in a distribution system (Van der Kooij, 1992). However, DOC can be measured before and after an incubation with indigenous bacteria, in order to measure biodegradable DOC (BDOC) as an alternate method of nutrient measurement. Reduction of BDOC has been shown to decrease bacterial numbers in a distribution system after disinfection depletion (Servais et al, 1993). AOC measurements may underestimate growth potential, since they are based on a two species inoculum, and are consistently lower than BDOC measurements (APHA, 1992). BDOC may serve as a more accurate assessment of growth potential.

Nutrient limitation in a water distribution system may not necessarily take the form of carbon limitation. One study has reported a system containing high levels of organic carbon which appeared to be phosphorous limited (Miettinen et al, 1996).

Indicator and pathogen detection and enumeration methods

The use of selective media

Traditionally, bacterial isolation and identification has been performed using using selective media, relying upon the target cell's ability to form colonies upon it. This practice

originated with clinical microbiology for diagnosis of pathogenic bacteria, and was subsequently extended to environmental health microbiology, including drinking water microbiology. Plate counts are one of the standard methods (APHA, 1992) of enumerating the indicator groups of coliforms, using m-Endo medium for "total coliforms" and m-FC medium for fecal coliforms following concentration of the bacteria from a water sample by membrane filtration. This method takes about 24 to 48 hours, is reasonably specific, can be very sensitive, inexpensive, is familiar to water industry workers and is able to provide historical background data.

This is not to imply this methodology is without faults. Due to the processing time required, the test is a retrospective indication of potential contamination. Potential relevance of the results can be of limited value, for reasons given elsewhere in this review. The test is also underpinned by measuring the cells' culturability rather than viability or infectivity.

Reduction of culturability seen in some waterborne bacteria can be compensated for by use of appropriate culture media. An example of this is the use of mT7 medium for coliform isolation from chlorinated waters (LeChevallier et al, 1983). This is a less restrictive medium than m-Endo or m-FC and allows recovery and growth of injured coliforms, giving a more accurate representation of their numbers.

The recent development of chromogenic presence/absence tests for coliforms (APHA, 1992) has provided greater simplification and standardization of microbiological water testing. These media rely upon the selective growth of bacteria and the production of indicative enzymes such as β -galactosidase and β -glucuronidase that catalyze a color

change or fluorescence within 24 hours. This methodology has potential drawbacks in generating a non-quantitative result unless performed in a multiple tube fashion. However, it is easier to perform than membrane filtration, has forced a more standardized sample volume on the U.S. water industry, and is more applicable to automation and on-line sampling.

Rapid on-line monitoring of water supplies is very desirable in the control of a contamination event. With almost continuous automated sampling and rapid results, corrective action might be taken before contaminated water reaches consumers. One potential approach to this is using electrical impedance monitoring (Colquhoun et al, 1995; Silley and Forsythe, 1996). The method involves adding sample water to a specific substrate medium, then monitoring electrical impedance during incubation. In this system, the growth of the target organisms gives a typical change of the impedance profile over time, within 14 hours for coliform detection (Colquhoun et al, 1995). The application of this technology to drinking water is still in the formative stages, but represents an interesting potential development for the industry. Impedance may be more rapid than other growth-based techniques, but still may be unable to deliver results in the time frame needed to prevent water reaching consumers.

Using selective plating for detection of bacterial pathogens has many of the same advantages and drawbacks as described for coliforms. In addition, larger sample sizes must be taken to allow for the usual low densities of these organisms even in contaminated waters. Detection methods often involve enrichment steps that can extend the time of analysis and lower the quantitative aspect of gathered data. Given the wide range of

potential bacterial pathogens, testing for them all by their various culture plating methods on a routine basis is impractical for even the best equipped water utility laboratory.

There exists no culture media test for detection of viruses or protozoa. The analogous traditional method of virus detection employs concentration methods usually based upon the surface adsorptive properties of the viral particles to harvest the particles from 400 litres or more of water. Detection is by performing a cytopathogenicity assay of the concentrate upon a host cell culture (Rao and Melnick, 1986; Hurst et al, 1989). These methods are limited by (1) the failure of some viruses to produce cytopathogenic effects, (2) lack of growth in cell culture, (3) long incubation time, and (4) a requirement for skilled laboratory staff. Detection of pathogenic protozoa is dependant on non-culture methods such as immunofluorescence.

Alternative detection approaches to selective media

Perhaps the most readily applicable alternative detection methodology to selective culturing is that of immunofluorescent staining followed by direct microscopy. Field testing this method for enteropathogenic *E. coli* detection in water samples has been reported as far back as 1960 (Bohloul and Schmidt, 1980). Today, antibodies are available to a wide range of epitopes from pathogenic organisms, and have wide applications in ecological studies and routine confirmation of food safety. As outlined by Bohloul and Schmidt (1980) there remain some limitations with using this method. These are (1) antibody specificity and nonspecific staining; (2) autofluorescence or nonspecific adsorption to background; (3) antigen stability under different growth conditions and

environments; (4) distinguishing between live and dead cells; and (5) efficiency of cell recovery of samples for quantification. These limitations apply to any application of immunofluorescence methodology.

Antibody detection of *E. coli* O157:H7 in water has been recently investigated by Pyle, et al (1995). Immunofluorescence was combined with cyanoditoyl tetrazolium chloride (CTC) staining to detect viable cells; this procedure was able to be completed within 3-4 hours.

Another way to detect immunologic staining is by use of an enzyme-linked immunosorbent assay (ELISA). An example is the use of an enterobacterial common antigen in an ELISA assay to detect *Enterobacteriaceae* in drinking water samples (Hubner et al, 1992).

An alternate method for visualization of specific target cells is the use of fluorescently labelled oligonucleotide probes to bind with specific regions of cellular ribosomes. Given the advances in sequence databases over recent years, the target regions can be chosen to tailor specificity from kingdom level to individual strains. This has been applied previously to the analysis of microbial communities (DeLong et al, 1989; Ward 1989). The method has some of the same limitations as antibody staining, although targeting ribosomes with a relatively small molecule has advantages in the stability of the target molecule, and ribosome numbers can reflect the physiological status of individual cells. Model drinking water biofilms have been examined using this method to determine general bacterial groups (Manz et al, 1993) and to detect pathogenic *E. coli* (Szewzyk et al, 1994).

A limitation of using fluorescent labelling to detect cells is the direct microscopy time required to examine samples for target organisms. This can be alleviated with the use of the flow cytometer, an automated microscope which examines a continuous stream of sample with respect to particle properties and previous labelling with fluorochromes. Modern flow cytometers are also capable of sorting suspect particles such as bacterial cells for further examination. This type of automation can radically cut subjectivity and labour involved with microscopic examination. This seems particularly relevant when examining large sample volumes for very low numbers of target organisms (Vesey et al 1993a and 1993b).

The rapid evolution of the polymerase chain reaction (PCR) as an analytical technique over the past decade offers much promise as a microbial detection method. Correct choice of primers can give specificity of amplification as desired, including that of nonculturable as well as nonviable organisms. Studies using spiked samples of drinking water have indicated excellent sensitivity (Bej et al, 1991a and 1991b; Kapperud et al 1993; LeChevallier et al 1994), often to the detection of a single cell. Processing can be "rapid", giving accurate results with a low cost within a few hours. Despite this potential, problems still remain in application of this technology as a primary detection method for environmental samples. Concentration of the necessary large sample volumes while eliminating environmental substances known to be inhibitory to the polymerase enzyme, all in a timely and cost-effective method, still remains a major problem. Optimization of reactions including cycling temperature protocols and magnesium ion concentration is crucial, along with strict quality control. Another consideration is the significance of

detected DNA, and whether it comes from viable or nonviable organisms.

An immediate application of PCR is as a bacterial identification methodology. Given currently available primer sets and thermal cycler technology, a suspect colony from selective medium can be prepared, processed and examined by gel electrophoresis in under 2 hours to give a definitive identification (unpublished data). This approach has been taken by other investigations when source water has required an enrichment step prior to PCR of the sample (Way et al 1993; Kapperud et al 1993).

Another methodology showing potential for future application is the profiling of extracted fatty acid methyl esters (FAMES) of bacterial cells using gas chromatography and mass spectroscopy (GC/MS). This has been applied successfully for the identification of colonies grown under specific conditions, using the Microbial ID Inc. (MIDI) system. The method involves comparison of the isolate's whole-cell GC/MS FAME profile to a database of known profiles. Some work has been performed in applying this methodology to FAME profiles of microbial communities rather than individual isolates (Haack et al, 1994). In this investigation, specific signature FAMES or ratios of key groups of FAMES were used to indicate the presence of target organisms. This method currently appears to be of most use in assessing the relative similarities and differences of communities with respect to their constituent organisms. Detection of specific organisms depends on growth conditions for quantities of specific cellular FAMES. In the case of pathogens such organisms can be numerically minor members of a community, and the signature FAMES thus only present in minute quantities.

Determination of the physiological status of a cell by means other than selective

plating gives much information as to its behaviour in an environment. Previous studies have made use of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) as an indicator of respiration (Rodriguez et al, 1992; Schaule et al, 1993; Pyle et al 1995), although this may have limited application with drinking water if CTC and chlorine interact (Morin and Camper, 1996). An alternative measurement can be performed using rhodamine 123, which is selectively accumulated by viable cells (Kaprelyants and Kell, 1992).

STATEMENT OF PROBLEM

Little is known about the capacity of specific pathogens to persist, survive or grow within a drinking water distribution system. This project attempts to provide qualitative information on the behaviour of selected bacteria within such a system, based upon observations of a laboratory-scale reactor seeded with test organisms.

It is apparent that different behaviours can be expected from different bacteria under oligotrophic drinking water conditions. This led to the selection of a range of bacteria for the experimental work. Two outright enteric pathogens, *Salmonella typhimurium* and *Escherichia coli* O157:H7, were selected on the basis of their previous history of causing significant waterborne outbreaks of disease. Two potential opportunistic pathogens, *Aeromonas hydrophila* and *Klebsiella pneumoniae*, were selected due to their frequent isolation from distributed water, and the associated arising regulatory concerns and potential hazards for the immunocompromised population.

Physiological changes caused by exposure to the water distribution system environment mean that detection of the tested organisms must be accomplished by methods reliant on criteria other than culturability. This led to the use of a variety of methods, including immunofluorescence, total cell counts, viable counts, and the polymerase chain reaction in the experimental phase of the project, supplemented by and compared with selective media.

Bacterial survival within the system appears to be heavily influenced by activities connected to the biofilm. Ease of biofilm examination was accomplished by the use of

laboratory-scale reactors. The use of laboratory reactors avoided the logistical problems of determining pathogen persistence or proliferation in a field-scale system, as well as allowing easy control of other environmental variables.

MATERIALS AND METHODS

The objective of this research was to examine the behaviour of some bacteria often associated with drinking water or with waterborne disease, under conditions relevant to a drinking water distribution system as simulated in an annular reactor. The bulk phase and the biofilm of experimental reactors were examined by culture plating and by immunofluorescent staining.

Test organisms

The following organisms were used in experimental runs.

Klebsiella pneumoniae (New Haven, Connecticut; drinking water isolate).

2 *Aeromonas hydrophila* isolates (Sydney, Australia; drinking water isolates).

Salmonella typhimurium (Gideon, Missouri; waterborne outbreak strain, kindly provided by Eugene Rice of USEPA, Cincinnati).

Escherichia coli O157:H7 (Clinical isolate; kindly provided by Gordon McFeters and Barry Pyle of Montana State University, Bozeman).

Experimental system

Experimental work has been performed in two laboratory polycarbonate annular reactors (RTs or Rototorques). These reactors have twelve removable polycarbonate

slides to allow for periodic biofilm sampling. The rotation of the inner cylinder was set to simulate a shear stress in a 10cm pipe of 0.3 m s^{-1} . The wetted surface area for each reactor is 0.19 m^2 with a total liquid volume of 0.6 L . Test conditions were selected to represent actual spring-summer distribution system conditions of high temperature and nutrients, which is when regrowth events are most likely to occur. Reactors had various influent feeds as described below.

(1) An assimilable organic carbon (AOC) feed jug, prepared by addition of filter-sterilized stock solution to autoclaved reverse osmosis (RO) water. This was fed to the reactor at 0.25 mL min^{-1} , therefore diluted 20-fold, yielding a final concentration of 0.5 mg L^{-1} of carbon in the reactor, giving carbon-limited conditions. The AOC solution consisted of equimolar concentrations on the basis of carbon of sodium acetate, sodium benzoate, absolute ethanol, parahydroxybenzoic acid, and propionaldehyde, as shown in Figure 1. Ferrous sulphate was added to ensure iron was not limiting.

Figure 1: AOC solution component concentrations.

Compound	Stock concentration	Feed Jug concentration	RT concentration
Sodium acetate	0.342 g L^{-1}	$68.4 \text{ } \mu\text{g L}^{-1}$	$3.42 \text{ } \mu\text{g L}^{-1}$
Sodium benzoate	0.172 g L^{-1}	$34.4 \text{ } \mu\text{g L}^{-1}$	$1.72 \text{ } \mu\text{g L}^{-1}$
Absolute Ethanol	0.242 mL L^{-1}	48.4 nL L^{-1}	2.42 nL L^{-1}
p-Hydroxybenzoic acid	0.164 g L^{-1}	$32.8 \text{ } \mu\text{g L}^{-1}$	$1.64 \text{ } \mu\text{g L}^{-1}$
Propionaldehyde	0.2 mL L^{-1}	40 nL L^{-1}	2 nL L^{-1}
Ferrous sulphate	0.75 g L^{-1}	$150 \text{ } \mu\text{g L}^{-1}$	$7.5 \text{ } \mu\text{g L}^{-1}$

(2) A buffer feed jug, prepared by addition of filter-sterilized stock solution of sodium nitrate and potassium phosphate (dibasic and monobasic) to autoclaved RO water. This solution was fed to the reactor at 0.25 mL min^{-1} , therefore diluted 20-fold yielding a final concentration of 0.1 mg L^{-1} of nitrate and phosphate in the reactor. Buffer composition is shown in Figure 2.

Figure 2: Buffer solution component concentrations

Compound	Stock concentration	Feed Jug concentration	RT concentration
Sodium nitrate	10 g L^{-1}	2 mg L^{-1}	0.1 mg L^{-1}
Potassium phosphate db	7 g L^{-1}	1.4 mg L^{-1}	0.07 mg L^{-1}
Potassium phosphate mb	3 g L^{-1}	0.6 mg L^{-1}	0.03 mg L^{-1}

(3) A dilution water influent, consisting of non-sterile RO water, fed at a rate of 4.5 mL min^{-1} . This source provided the undefined mixed heterotrophic bacterial population in the experiments at a relatively constant $\sim 10^4 \text{ CFU mL}^{-1}$ (determined by R2A enumeration).

(4) A disinfectant solution, containing 1.0 or 4.0 mg L^{-1} of free chlorine, was made by adding commercial bleach (Clorox) to non-sterile RO water, then ensuring final concentration was correct with a free chlorine assay (LaMotte DC1100 digital colorimeter and test kit). This solution was fed after establishment of the biofilm, usually about 2 weeks into an experimental run. Chlorination was started at the lower level and then raised to the higher level after about a week. The feed jug solution was pumped to the reactors at a rate of 0.25 mL min^{-1} , and diluted 20-fold to give 0.05 or 0.2 mg L^{-1} influent free

chlorine. Dilution water inflow was lowered to 4.25 mL min^{-1} once disinfection solution flow was begun, to keep the overall flow rate constant.

These inflows gave a total input to the reactors of 5 mL min^{-1} , for a residence time of 2 hours. Previous experiments (Camper, 1995) have shown this residence time is sufficiently short so that the vast majority of biological activity is within the biofilm and not in the bulk fluid. Temperature was maintained at approximately 20°C by a refrigerated water circulator linked to a heat exchange coil immersed in a water bath, in which the reactors were also immersed.

Where possible, reactors were run in duplicate, with one being chlorinated as described above and the other serving as an unchlorinated control. This was not possible for every experimental run; such situations are noted in the results section.

Experimental runs were begun by streaking the target organism from frozen stock cultures onto R2A agar (Difco), and incubating for 2 days at room temperature. This culture was used to inoculate a shaker flask containing 100 mL of sterile water with $5000 \mu\text{g carbon L}^{-1}$ of AOC plus $100 \mu\text{g L}^{-1}$ nitrate and phosphate of the same composition as described above for the AOC and buffer influent feeds. After 3 days incubation at room temperature, the contents of this shake flask were used to inoculate the reactor. The annular reactor was run as a chemostat (no rotation of the inner drum) with only the AOC and buffer feed inputs, at a total feed rate of 0.5 mL min^{-1} . Stocks were therefore diluted 2-fold rather than 20-fold, to give a final concentration of $5000 \mu\text{g carbon L}^{-1}$ of AOC and $1000 \mu\text{g L}^{-1}$ of nitrate and phosphate. The reactors were run in this fashion for 3 twenty hour residence times, or 60 hours. The dilution water influent was then introduced, along

with its continuous input of heterotrophs, and shear stress equivalent to flow of 0.3 m s^{-1} in a 10cm pipe imparted by rotating the inner cylinder. This was maintained through the experiment.

Sampling protocol

The first samples (designated Day 0) were collected 3 days after introduction of dilution water. Samples were taken every 5-7 days, as shown in the individual runs (Appendix A), with sampling of the reactor effluents and biofilms.

Effluent samples of up to 150 mL were collected. Free chlorine was analyzed (LaMotte DC1100 digital colorimeter and test kit) if this disinfectant was being fed to the reactor. The sample was homogenized at 24000 rpm for 30 sec (Tekmar Tissuemizer with UT-dispersing tool T25 type S25N-8G) to disperse clumped bacteria. If target organism numbers were expected to be low, volumes over 0.5 mL were filtered through $0.2\mu\text{m}$ pore size 45mm diameter nitrocellulose filter membranes (Whatman), which were then transferred to the appropriate agar plate. Otherwise, volumes of 0.01 mL to 0.5 mL were spread directly on the agar plate. For volumes below 0.01 mL, serial dilutions using sterile water were prepared. Aliquots for microscopy were filtered through $0.2\mu\text{m}$ pore size, 20mm diameter black polycarbonate filter membranes (Poretics).

Biofilm samples were taken by removing a polycarbonate slide from the reactor using sterilized pliers. The bottom 5cm of the slide was cut off using sterilized scissors for fluorescent antibody staining of intact biofilm. The remainder of the slide (consisting of

25cm² surface area) was suspended in 5mL of sterile water, and scraped using a sterilized utility knife or razor blade. The solution containing the resuspended cells was then homogenized (Tekmar Tissuemizer) as described above. Aliquots were prepared for microscopy or transferred to relevant media, using spread plating, serial dilution and membrane filtration as for the effluent samples.

Samples for immunofluorescence along with DNA staining were fixed overnight in a 1% formaldehyde (Fisher) solution, prepared on the day of sampling. The staining protocol was as described below in the analytical methods section below.

Rhodamine 123 samples were stained immediately as described in the analytical methods section below.

Analytical methods

Culturing techniques

The heterotrophic bacterial populations were enumerated on duplicate R2A agar (Difco) plates. Two sets of plates per sample were prepared, with incubation for one set at 35°C for 48 hours, and the other at room temperature for 7 days.

Klebsiella samples were plated on m-Klebsiella (mKA) agar (APHA, 1992) and on m-T7 agar (APHA, 1992), and incubated 24 hours at 35°C.

Aeromonas samples were plated on m-Aeromonas (mAA) agar (Oxoid) and on Ampicillin Dextrin (ADA) agar (Havelaar et al, 1987), and incubated 24 hours at 30°C.

Escherichia coli O157:H7 samples were plated on m-T7 agar (APHA, 1992), and

incubated 24 hours at 35°C.

Salmonella samples were plated on xylose lysine desoxycholate (XLD) agar (Difco), and incubated 24 hours at 35°C. Samples of equal volume were also inoculated into tetrathionate broth (Difco) and incubated 35°C, from which 0.1 mL was taken at 24 and 48 hours and streaked on XLD, which was then incubated at 35°C for 24 hours.

Media were prepared as per manufacturers instructions, or to referenced instructions.

All plates examined at 24 hours were reincubated and reexamined after another 24 hours for further growth.

Positive controls for all selective plating were performed by streaking a culture isolate of the test organism on the appropriate selective medium. Negative controls for all selective plating were performed by plating the reactor dilution water on the appropriate selective medium in every sampling run. Confirmation of suspected test organisms was found to be generally unnecessary, as colony morphology compared with positive and negative controls was found to be definitive in most cases. In some cases, suspect colonies during *Aeromonas* runs were confirmed using PCR.

Microscopy techniques

All microscopy was performed using an inverted confocal scanning laser microscope (Biorad MRC600), plus image analysis software (COMOS) and a 60X oil immersion objective with numerical aperture of 1.4 (Olympus). Detection of fluorescein isothiocyanate (FITC) labelled antibody staining was performed using a "K2" filter block,

which detects light emissions of 522-560 nm. This was performed simultaneously with propidium iodide (PI) staining, which was detected using a "K1" filter block, with a detection range of >560nm. Rhodamine 123 staining was detected using a "BHS" filter block, with a detection range of >515 nm. Sample preparation by staining is described below in "Staining techniques".

Only the membrane filter mounted samples were used for the purposes of numeric data acquisition. Three 105µm by 130µm overlaid images of antibody staining and propidium iodide staining were taken of each sample. Cells were then counted from the three images, averaged, and multiplied to equate to the filter surface area examined. Total cell count subjects were selected on the basis of strong propidium iodide staining plus dimensions and shape resembling a bacterial cell. Target antibody cell counts had to have both the propidium iodide staining in a cell shape, and definite staining on and around the cell with labelled antibody. This added a subjective element to the cell counts. Positive antibody stained controls of the target organism suspended in nanopure water and filtered to a membrane, and negative antibody staining controls of membrane filtered reactor dilution water were performed with each sampling run.

Rhodamine 123 microscopy consisted of taking the average count from ten 210µm by 130µm images and multiplying to equate to surface area examined. Positive staining controls of reactor dilution water filtered to a membrane were performed with each sampling run.

Staining techniques

Primary and labelled secondary antibodies were initially tested on fixed samples of pure cultures of test organisms to determine the appropriate antibody dilution.

Antibodies used in this study included the following:

(1) Primary antibodies:

Rat *Klebsiella pneumoniae* monoclonal specific for the New Haven isolate (Stewart et al, 1996), kindly provided by Anne Camper, prepared by MSU veterinary science unit.

Rat *Aeromonas hydrophila* polyclonal, kindly provided by Garth James.

Goat *Escherichia coli* O157:H7 monoclonal, purchased from Kirkegaard-Perry Inc.

Goat *Salmonella typhimurium* monoclonal, purchased from Kirkegaard-Perry Inc.

(2) Fluorescein isothiocyanate (FITC) labelled secondary antibodies:

Goat anti-Rat and Rabbit anti-Goat monoclonal antibodies purchased from Fisher.

Double staining was performed on one set of microscopy samples. This was performed with the cellular DNA stain propidium iodide, to identify and enumerate intact bacterial cells, and with labelled antibodies to identify and enumerate target organisms.

Staining of cells began by fixing cells to a black polycarbonate membrane or a polycarbonate slide using formaldehyde. The samples were initially washed 3 times with Tris-buffered saline or TBS (9.0 g L⁻¹ sodium chloride - Fisher, 1.2 g L⁻¹ trizma base - Sigma in nanopure water, pH 7.4). Blocking solution (TBS with 5 mL L⁻¹ Tween80 - Sigma and 10 g L⁻¹ powdered skim milk - Lucerne), was applied for 30 minutes and samples were washed 3 times with TBST (TBS with 500µL L⁻¹ Tween80). Primary antibody diluted in TBST was applied for 90-120 minutes and samples were washed 3

times with TBST. Labelled secondary antibody diluted in TBST was applied for 90-120 minutes, followed by washing 3 times in TBST. Proidium iodide (Sigma) diluted in TBST to $50 \mu\text{g mL}^{-1}$ was applied for 30 minutes and samples were then washed once in TBS previously adjusted to pH 9, dried, and examined microscopically.

Samples for rhodamine 123 staining, using the second set of samples, were first permeabilized for 5 min in a 50mM Tris (Sigma), 5mM EDTA (Baker), pH 8 buffer (TE buffer). Rhodamine 123 (Kodak) was then added to give a final concentration of $5 \mu\text{g mL}^{-1}$, the solution was left in the dark at room temperature for >1 hour. Samples were then rinsed 3 times with TE buffer, dried using a membrane filter apparatus, and oil mounted for microscopic examination.

A nonselective enrichment procedure was performed with some of the *Salmonella* samples prior to antibody staining in order to allow viable cells to grow. This consisted of covering a section of a reactor slide with an enrichment solution (0.4 g L^{-1} brain heart infusion - Difco, 0.1 g L^{-1} glucose - Sigma in nanopure water, autoclaved) in a sterile petri dish, and incubating for 3 days at 35°C . The enrichment medium was then filtered through a $0.2\mu\text{m}$ nitrocellulose membrane and transferred to XLD. The XLD results allowed a presence - absence indication for viable *Salmonella*. The slide sections were then fixed with 1% formaldehyde, and stained using fluorescently labelled antibodies and propidium iodide as described above. Microscopic examination was performed to detect development of *Salmonella* microcolonies on the slide.

PCR techniques

The PCR primer sets used were kindly provided by Peter Cox of Australian Water Technologies, Sydney and Matthias Dorsch of the University of New South Wales, Sydney. Thermal cycler use and lab space were kindly provided by Dave Ward of Montana State University, Bozeman.

PCR was used as an identification tool for suspect colonies from selective media, and for positive confirmation of the original isolates of *Salmonella* and *Aeromonas hydrophila*. Target colonies were transferred to thermal cycling tubes containing 100 μ L of TE/Triton (10mM Tris, 1mM EDTA, 0.01% Triton X100, pH 8). This was boiled in the thermal cycler for 15 min, then 5 μ L was taken and added to another thermal cycling tube containing 45 μ L reaction mix (1x buffer, 1.5mM MgCl₂ for *Aeromonas* and 2.5mM for *Salmonella*, 1mM dNTPs, *Taq* polymerase, primers as described below) using reagents from a PCR Core Kit (Boehringer-Mannheim). In all cases a positive control from an R2A plate colony, and an uninoculated negative control were amplified simultaneously with the test samples. This was performed as described below.

The *Salmonella* primers used were the ST11/ST15 set, complementary to regions within a 2.3 kb *Salmonella*-specific DNA region, as described by Aabo et al (1993). These amplify a 429 bp fragment. The *Aeromonas hydrophila* primers used were from Dorsch et al (1994), specific for positions 450 to 472 of the 16S rDNA sequence, with the general *Aeromonas* primer for position 1115 to 1135, resulting in a 685 bp fragment. No primers were used for *Klebsiella* or *E. coli*, as these organisms were not confirmed using this method.

Thermal cycling protocols were as follows.

Aeromonas: 80°C for 30 sec, 95°C for 10 sec for initial denaturation. This was followed by 30 cycles of 93°C for 30 sec for denaturation, 55°C for 30 sec for annealing, 72°C for 60 sec for extension, followed by 72°C for 5 min for final extension, reactions then cooled to room temperature.

Salmonella: 30 cycles of 94°C for 1 min for denaturation, 57°C for 1 min for annealing, 72°C for 2 min for extension, 10 min for the last cycle for final extension, as described by Aabo et al (1993).

Twenty microliters of PCR products were run on a 1% agarose flat gel in Tris-Acetate-EDTA buffer with glycerol-bromothymol blue loading buffer, along with a lambda-digest size ladder. When marker dye was about 2/3 of way down the gel, it was stained with ethidium bromide and examined under UV light. Positive amplification resulted in single bands visible on the gel.

Data analysis

Much of the data gathered resulted in values below detection. For numeric purposes, these values were converted to half of the value it was less than. For example, a count of <1 cell/mL was converted to 0.5 cells/mL. This detection threshold varied between experimental runs, and was dependant upon the exact volume or area examined.

Data series were examined for the effects of chlorination upon target cell numbers. This required a steady state to be apparent before chlorination, as no allowance was made

for the time of sampling in these calculations. Data points were divided into data sets of the logarithms of raw values from unchlorinated reactors, from those being chlorinated with 0.05ppm chlorine, and those with 0.2ppm chlorine influent. Influence of chlorine was shown by log reduction of counts, calculated by subtraction of the mean of chlorinated values from the mean of unchlorinated values. Error was calculated as the square root of the sum of the squares of the standard error from chlorinated and unchlorinated data sets.

In order to see variation between the heterotrophic and total counts of each run, data from unchlorinated sampling points were condensed according to source and detection method. Linear regression was performed upon each data series in order to generate a trendline, which was plotted on the same figure as trendlines from other runs, to allow a visual comparison of the similarity of the data.

To examine the effects of time in the reactor upon the heterotrophic and total bacterial populations, the same condensed data sets used to compute the trendlines were divided into time points, without differentiating between the original runs. As not all the sampling times were the same between experiments, some data points were grouped with nearby values; such groups were graphically represented by the median "Day" value. The means of these data sets were plotted, and error calculated as the standard error of each data set.

RESULTS

These results are composed of the data from fifteen experimental "runs", each a separate inoculation of an annular reactor. Five runs were performed using *Salmonella typhimurium*, four using *Escherichia coli* O157:H7, and three each with *Aeromonas hydrophila* and *Klebsiella pneumoniae*. The raw quantitative data from these runs is presented in graphical form in the Appendix (Figures 50 to 109). This data was condensed and presented in different ways in order to show trends and correlations within this section.

Heterotroph data

Heterotroph variability between runs

The heterotroph data have been examined for variability between the experiments. This was done to show if there was uniformity of reactor conditions between experiments, as it affects microbial growth and survival. In Figures 3-10, the raw heterotroph data from the unchlorinated reactors (Appendix) have been condensed according to source (bulk phase or biofilm) and detection method (2 day or 7 day incubation on R2A agar, propidium iodide staining, or rhodamine 123 staining). Linear regression has been performed on the data from each run, and a trendline plotted over the time when sampling took place in the run. This was in order to smooth out variation within the runs, and enable a visual comparison of the trend of heterotroph data between runs. No analysis of slope,

Figure 3: Heterotrophs in unchlorinated bulk phase by 2day counts - linear regression

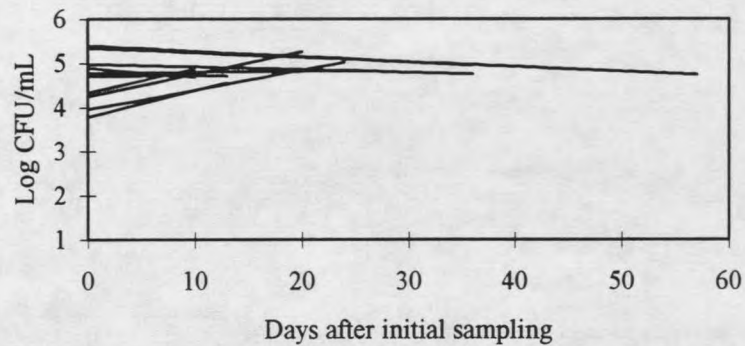


Figure 5: Heterotrophs in unchlorinated biofilm by 2day counts - linear regression

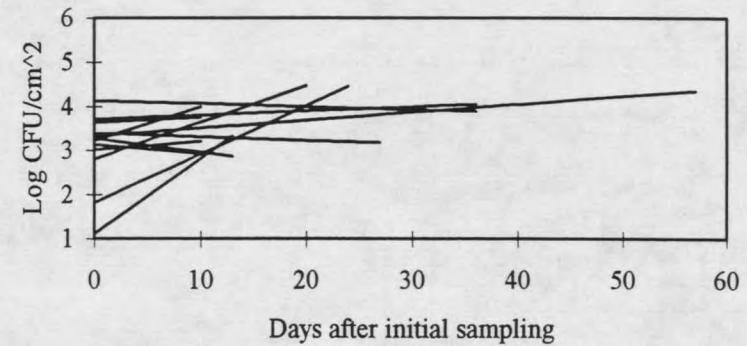


Figure 4: Heterotrophs in unchlorinated bulk phase by 7day counts - linear regression

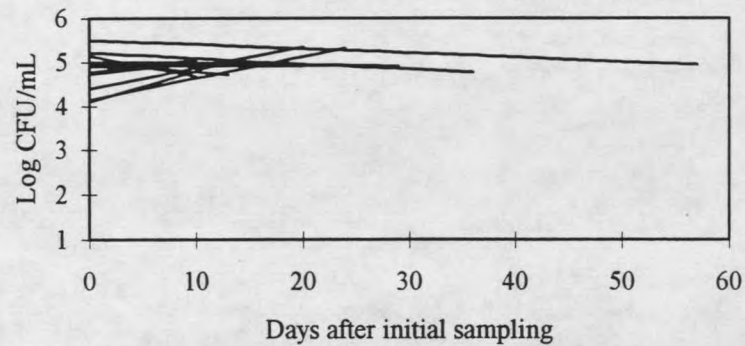
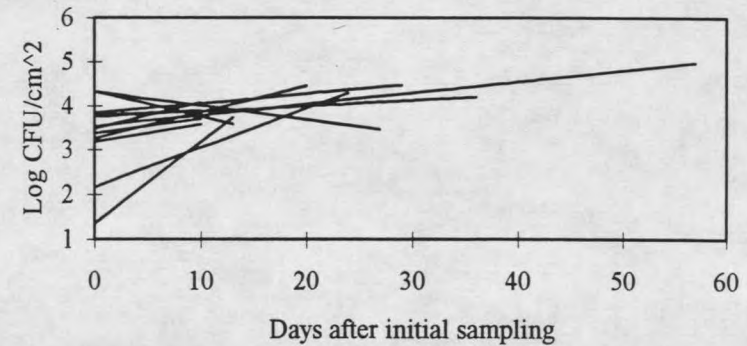


Figure 6: Heterotrophs in unchlorinated biofilm by 7day counts - linear regression



Trendlines calculated for each run over the unchlorinated period of sampling. "2day" and "7day" refer to 2 day and 7 day incubation on R2A agar.

Figure 7: Heterotrophs in unchlorinated bulk phase by PI counts - linear regression

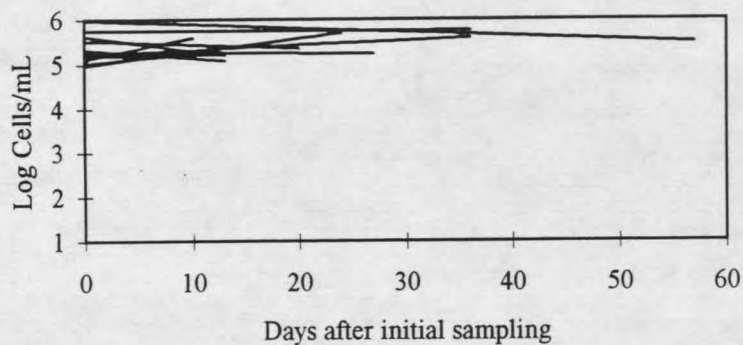


Figure 9: Heterotrophs in unchlorinated biofilm by PI counts - linear regression

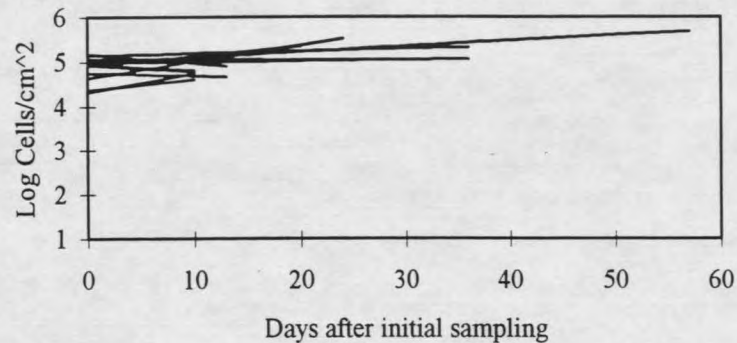


Figure 8: Heterotrophs in unchlorinated bulk phase by Rh123 counts - linear regression

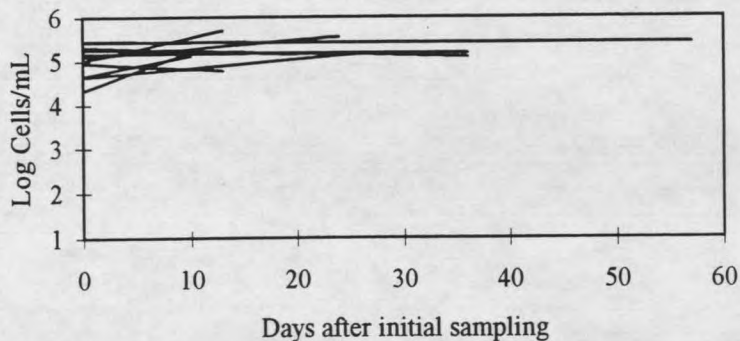
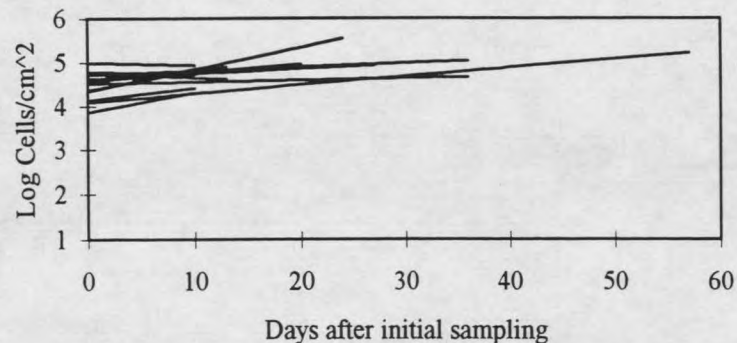


Figure 10: Heterotrophs in unchlorinated biofilm by Rh123 counts - linear regression



Trendlines calculated for each run over the unchlorinated period of sampling. "PI" refers to total count by propidium iodide. "Rh123" refers to viable count by rhodamine 123.

intercept or R^2 values was done.

The data from R2A enumeration with time appears to be similar from both 2 day and 7 day incubations, in both the bulk phase (Figures 3 and 4) and the biofilm (Figures 5 and 6). In the bulk phase, initial counts range between 4 to 5.5 log CFU mL⁻¹. The trendlines show counts converging on about 4.5 to 5 log CFU mL⁻¹, which was observed quite consistently through the experiments. Biofilm trendlines show a similar pattern originating from 3 to 4 log CFU cm⁻², although with greater initial variation than the bulk phase. This is not unexpected, since the substrata were initially uncolonized. These counts generally appear to increase over time, which is attributable to development of the biofilm, or to increased culturability of the cells.

The propidium iodide and rhodamine 123 counts appear to be similar to each other, in both bulk phase (figures 7 and 8) and biofilm (figures 9 and 10). The bulk phase counts show little variation between runs even at Day 0, ranging between 5 to 6 log cells mL⁻¹ with propidium iodide and 4.5 to 5.5 log cells mL⁻¹ with rhodamine 123. These levels were maintained through the course of the experiments. Biofilm counts for both these enumeration methods show levels of about 4 to 5 log cells cm⁻² at Day 0. Many of the trendlines appear to increase slowly from here, attributable to development of the biofilm.

Heterotroph behaviour in absence of disinfection

As the heterotroph data seem roughly comparable between runs, there would appear to be similar growth conditions between experiments. In order to better examine the behaviour of heterotrophs in the reactors over time in unchlorinated conditions, the

data used in Figures 3 to 10 are presented in Figures 11 to 18 as the mean of cell or colony counts over time. As not all the sampling times for all the experiments were on the same days, nearby days have been grouped, with the median value represented on the figure. Error bars represent the standard error of the group of counts.

It is evident that there is little variation in heterotroph numbers in the bulk phase of unchlorinated samples over the time-course of the experiments. This is shown in the constant numbers of cells or colonies over time in Figures 11 to 14. Figures 11 and 12 show the enumeration on R2A agar with 2 day and 7 day incubation, respectively. In both data sets, the mean count is slightly below $5 \log \text{CFU mL}^{-1}$ at Day 0, then remains constantly around $5 \log \text{CFU mL}^{-1}$. Enumeration using propidium iodide (Figure 13) and rhodamine 123 (Figure 14) show relatively constant counts between 5 and 6 log cells/mL throughout the experiments.

Figures 15 and 16 show the mean of biofilm heterotroph counts from R2A 2day and 7 day incubation, respectively. These are initially both around $3 \log \text{CFU cm}^{-2}$ at Day 0, then until Day 25 are seen at around $3.5 \log \text{CFU cm}^{-2}$ with 2 day incubation, and $4 \log \text{CFU cm}^{-2}$ with 7 day incubation. There is a rise in the mean 7 day counts on the last two data points, although this may be an artifact of relatively fewer experiments persisting to this length of time.

The numbers of biofilm cells enumerated by propidium iodide (Figure 17) and rhodamine 123 (Figure 18) appear relatively static over time. Propidium iodide counts remain around $5 \log \text{cells cm}^{-2}$ until about Day 20, then appear to rise slowly. Rhodamine 123 counts are somewhat noisy, but constant around 4.5 to $5 \log \text{cells cm}^{-2}$.

Figure 11: Mean unchlorinated bulk phase heterotroph values (2day R2A) over time

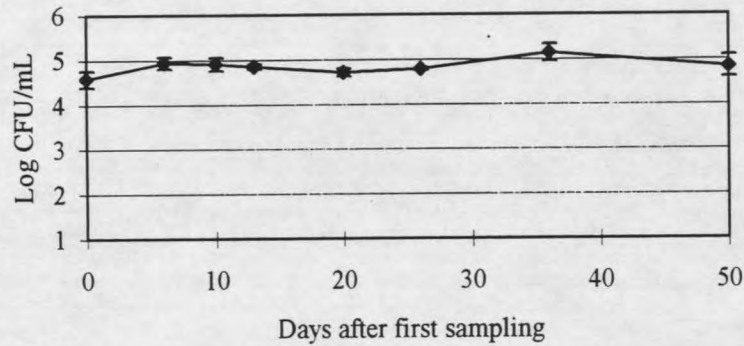


Figure 13: Mean unchlorinated bulk phase heterotroph values (PI) over time

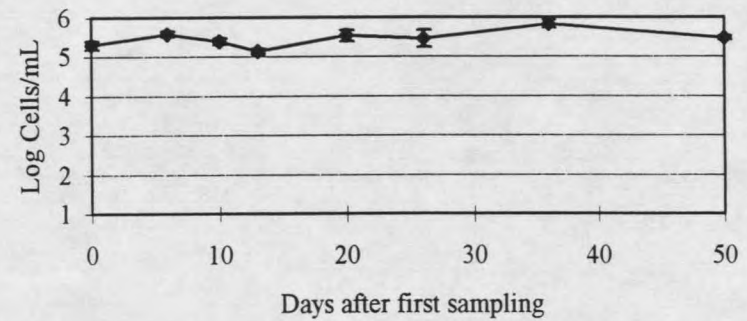


Figure 12: Mean unchlorinated bulk phase heterotroph values (7day R2A) over time

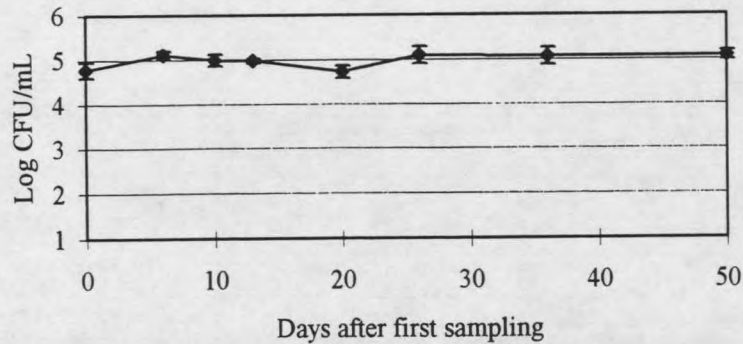
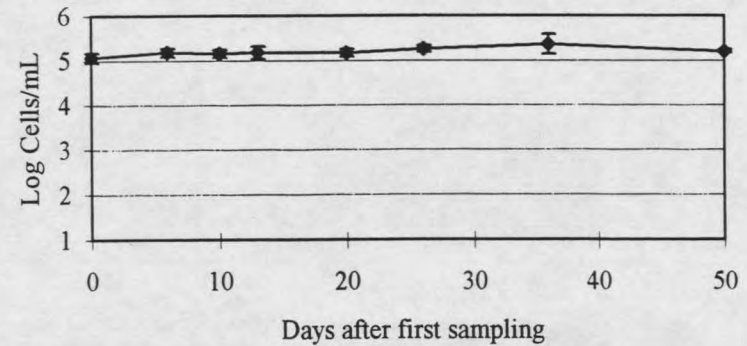


Figure 14: Mean unchlorinated bulk phase heterotroph values (Rh123) over time



Values represent mean of log unchlorinated values from all runs. Error bars represent the standard error of each data point. "2day" and "7day" refer to 2 day and 7 day incubations on R2A agar, "PI" to propidium iodide staining, "Rh123" to rhodamine 123 staining.

Figure 15: Mean unchlorinated biofilm heterotroph values (2day R2A) over time

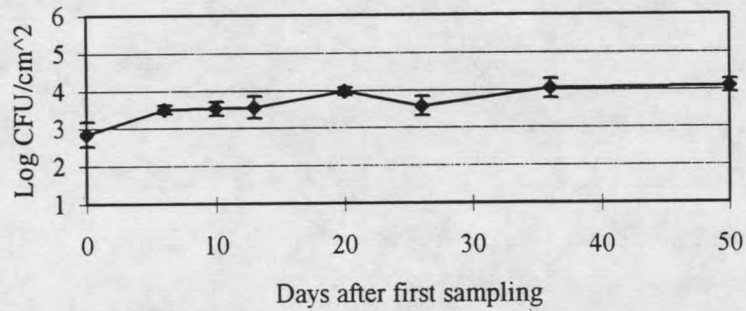


Figure 17: Mean unchlorinated biofilm heterotroph values (PI) over time

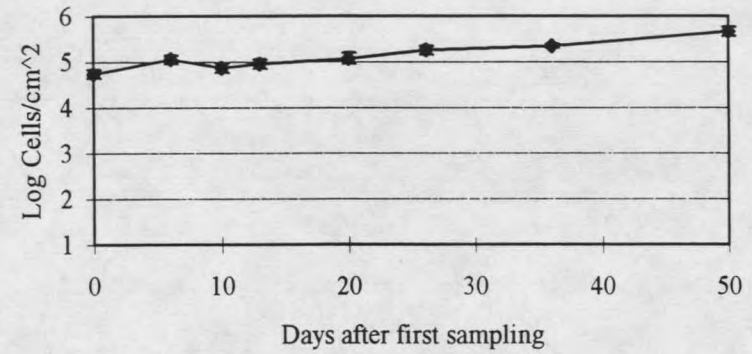


Figure 16: Mean unchlorinated biofilm heterotroph values (7day R2A) over time

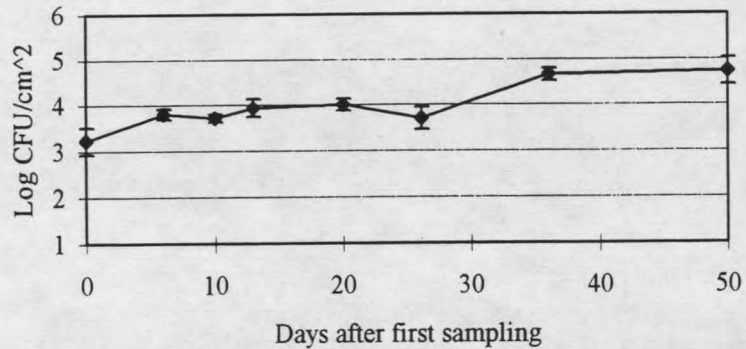
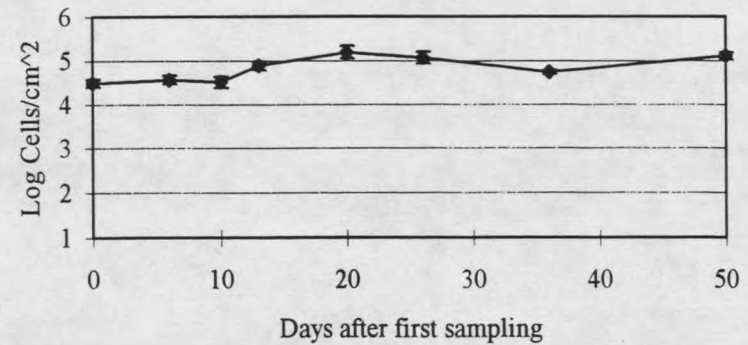


Figure 18: Mean unchlorinated biofilm heterotroph values (Rh123) over time



Values represent mean of log unchlorinated values from all runs. Error bars represent the standard error of each data point. "2day" and "7day" refer to 2 day and 7 day incubations on R2A agar, "PI" to propidium iodide staining, "Rh123" to rhodamine 123 staining.

Overall, it would seem that the heterotrophic and total bacterial population reached a pseudo-steady state within the bulk phase during the experiments. This was not the case with the biofilm, which exhibited slow growth. In both bulk phase and biofilm there was initial variation, which seemed to steady out within about two weeks. It was after this time point when chlorination was begun in some experimental runs, so relatively uniform starting conditions for chlorination can be assumed and comparison between runs is possible.

Heterotroph response to chlorination

Data summarized from all the experimental runs regarding the behaviour of the heterotrophic bacterial population in the reactor bulk phase and biofilm in response to chlorination is presented in Figures 19 and 20. Data sets were composed of the logarithm of the raw values from unchlorinated reactors, those being chlorinated with 0.05ppm chlorine, and those with 0.2ppm chlorine influent. All time points were averaged, only the disinfection regime used is considered here. Influence of chlorine is shown by log reduction of counts; calculated by subtraction of mean of chlorinated values from mean of unchlorinated values. Error bars represent the square root of the sum of squares of standard errors from chlorinated and unchlorinated data sets.

From Figure 19, it is apparent that 0.2ppm chlorination resulted in about a 3.5 log reduction in 2 day R2A counts, a 2 log reduction in 7 day counts, and virtually no effect on propidium iodide and rhodamine 123 values within the effluent samples. Thus, chlorination resulted in loss of culturability among much of the effluent bacterial

Figure 19: Chlorination effect on heterotrophs in bulk phase

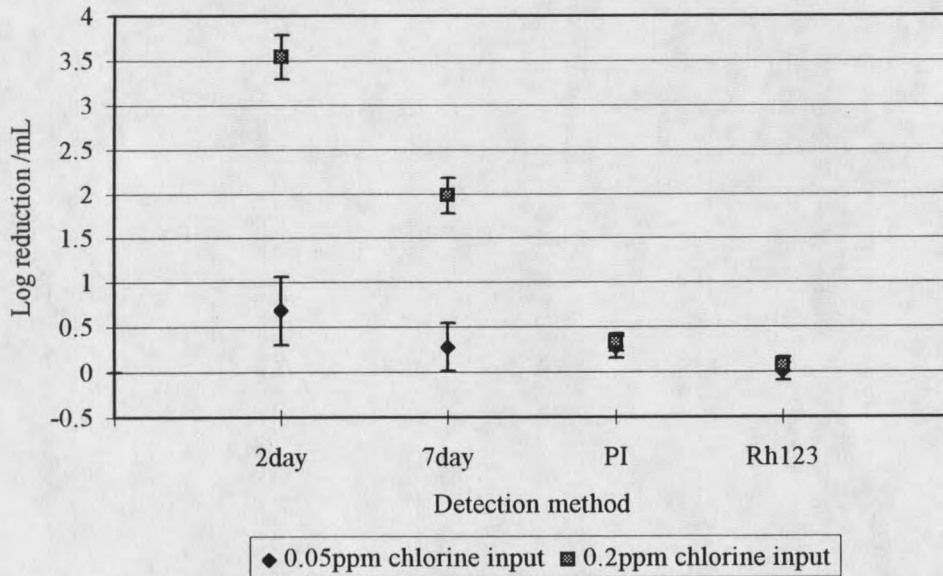
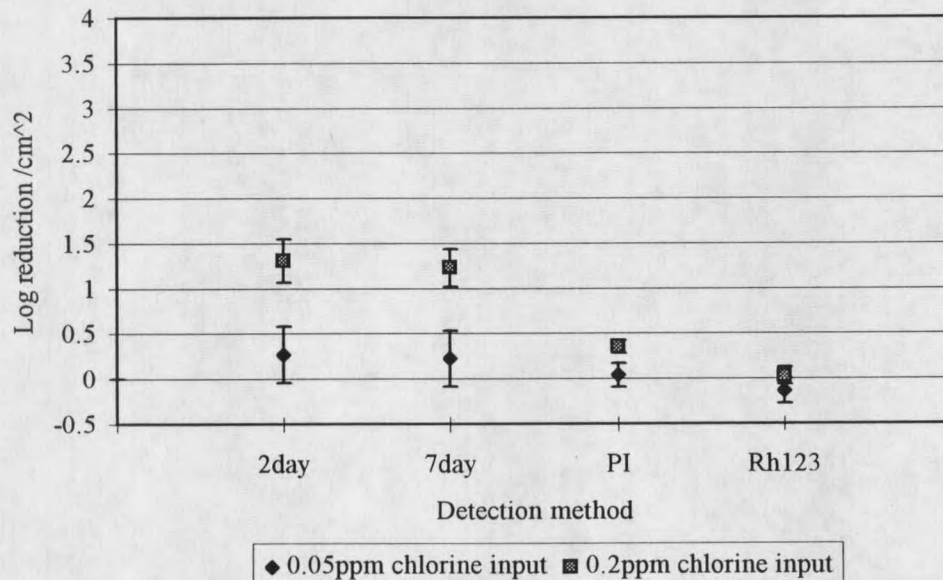


Figure 20: Chlorination effect on heterotrophs in biofilm



Log reduction calculated by subtraction of mean of log chlorinated values from mean of log unchlorinated values. Error bars represent the square root of the sum of squares of standard error from chlorinated and unchlorinated data sets. "2day" and "7day" refer to 2 day and 7 day incubations on R2A agar, "PI" to propidium iodide staining, "Rh123" to rhodamine 123 staining.

population, although total and viable counts remained largely unchanged. This observation is repeated with 0.05ppm chlorination, although the reduction in culturability is barely observed.

Not surprisingly, the biofilm population proved significantly more resistant to disinfection than the effluent population, as shown in Figure 20. Chlorination at 0.2ppm resulted in a 1 to 1.5 log cm^{-2} reduction of culturability with both 2 day and 7 day R2A incubation, and a slight (<0.5 log) reduction from 0.05ppm chlorination. Total and viable counts in the biofilm appeared unaffected by either treatment, as in the bulk phase. It is unclear why the biofilm heterotrophs enumerated in a 2 day R2A count would be as affected by chlorine as those in the 7 day count, while in the bulk phase the 2 day count population was markedly less than the 7 day count.

Test organism data

The data referred to in this section is again summarized from the Appendix. In the referred figures, the counts are noted as from appropriate selective media (XLD, mT7, mAA, ADA, or mKA) and from fluorescent antibody staining (Fab). Several of the data series have points where the counts are below the detection level; for numeric purposes this is converted to half the value that it is less than. For example, a count of <1 cell mL^{-1} is converted to 0.5 cells mL^{-1} . This detection threshold varies between experimental runs, and is dependant upon the exact volume or area of sample examined. It is noted graphically as a dotted horizontal line connecting data points. Introduction of chlorine to

individual reactors is noted by the use of gray-scale wedges. To avoid confusion, chlorine introduction is not noted if the parameter is already below detection level, this information is noted in the Appendix.

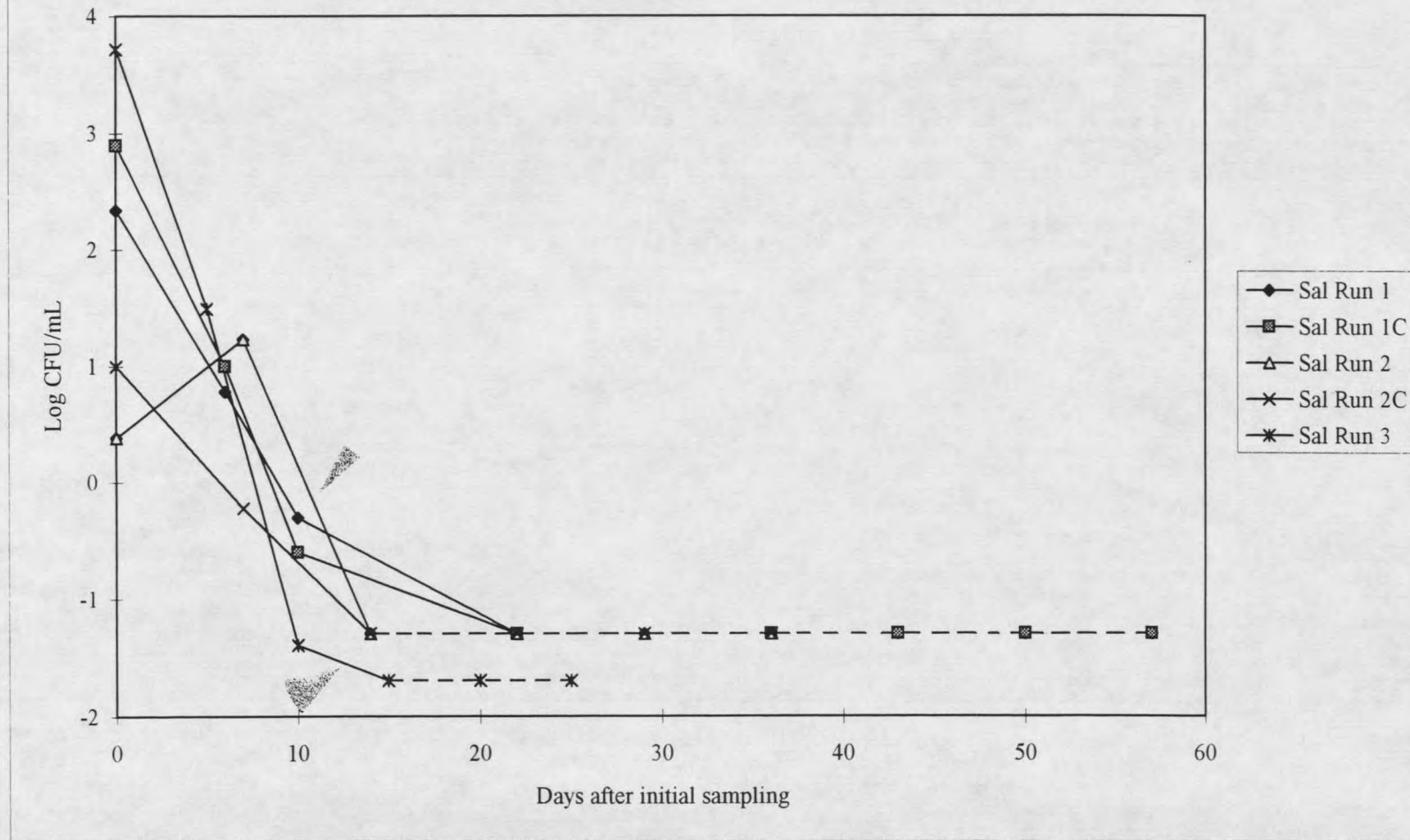
Salmonella typhimurium

Three chlorinated *Salmonella* test runs were performed during this project (Sal Runs 1, 2 and 3), along with two unchlorinated control runs (Sal Runs 1C and 2C), concurrent with Sal Runs 1 and 2. Data gathered using selective media and antibody staining for these runs has been summarized in Figures 21 to 24, with the individual data sets presented in the Appendix.

Figures 21 to 24 show the behaviour of *Salmonella* in the reactors. In all cases the cell counts from fluorescent antibody staining are higher than colony counts from selective agar XLD. The difference in these counts can be attributed to viable but nonculturable cells (VBNCs), injured cells, and dead cells in the reactor, as well as the antibody counts being close to the limits of detection using microscopy. This difference can be quite large, and is up to 5 orders of magnitude in some cases.

Results from selective plating on XLD showed a similar pattern in all runs in both bulk phase (Figure 21) and biofilm (Figure 22). There was a constant decline in plate counts from the beginning of sampling through to a non-detectable level within 10-15 days. Selective enrichment with tetrathionate broth followed by plating to XLD did not increase the detection of *Salmonella* compared to straight plating to XLD (data not shown).

Figure 21: Salmonella enumeration in bulk phase by XLD





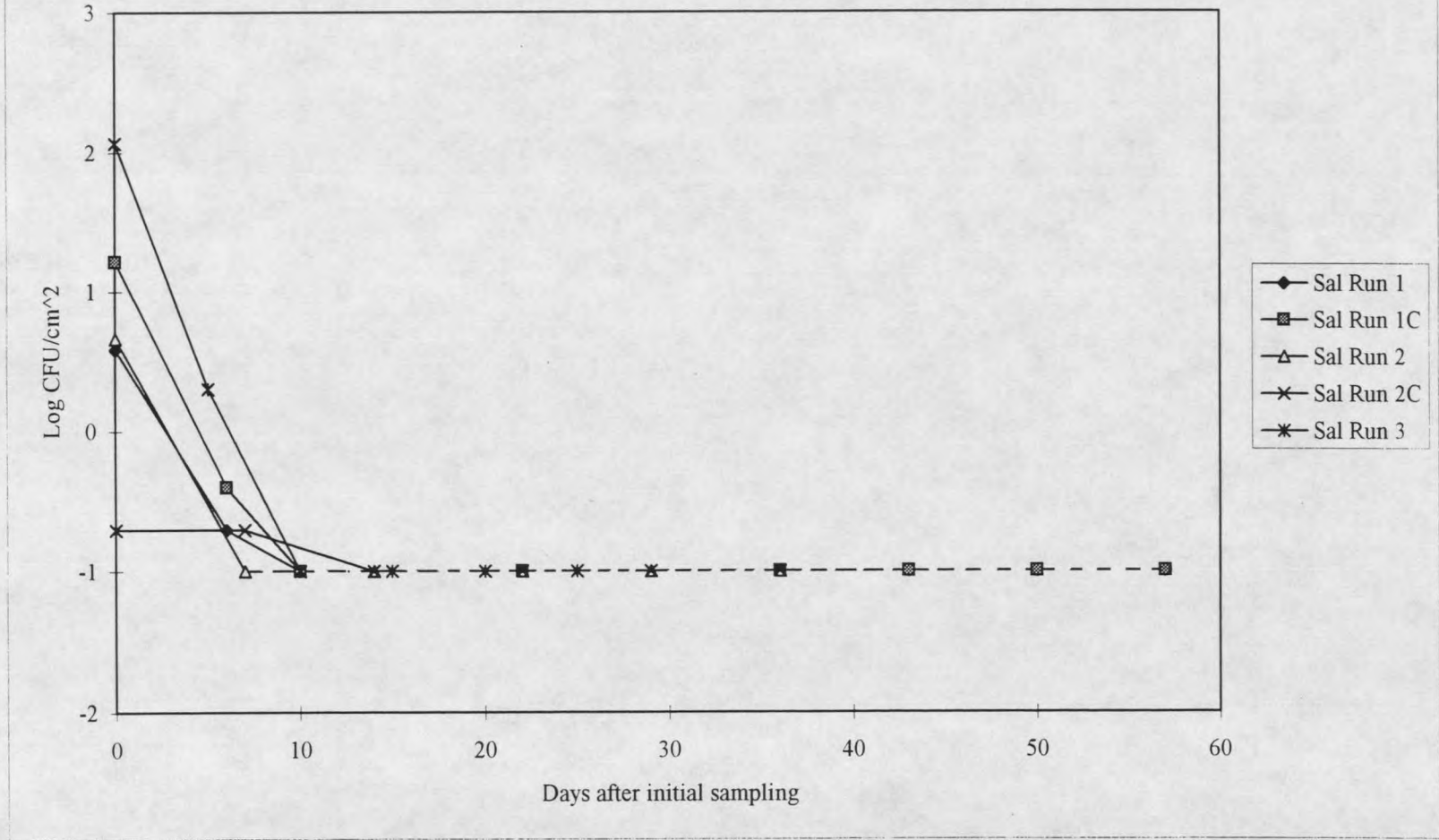
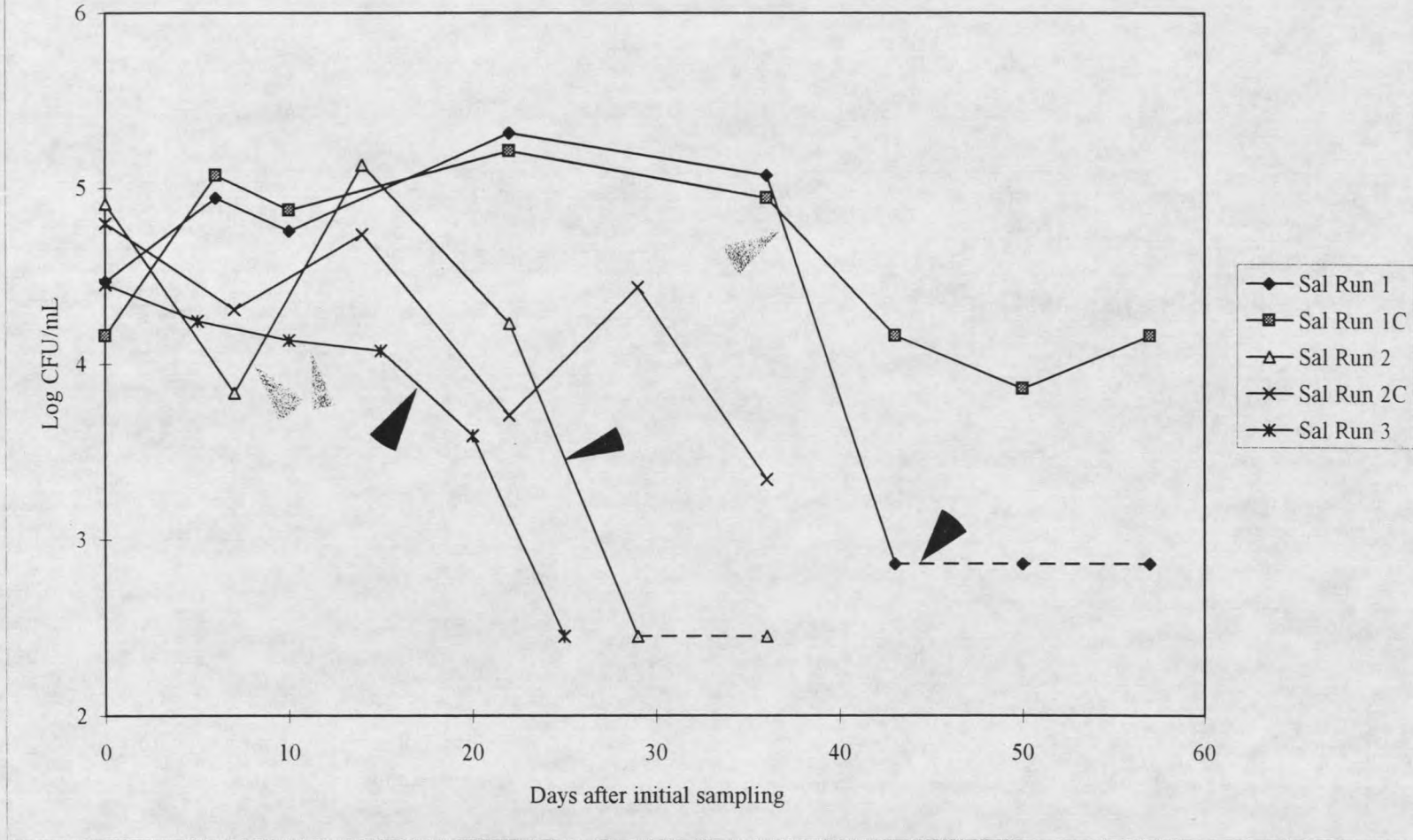
Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (). Chlorination at 0.2ppm is indicated by a dark gray wedge ().

Figure 22: Salmonella enumeration in biofilm by XLD



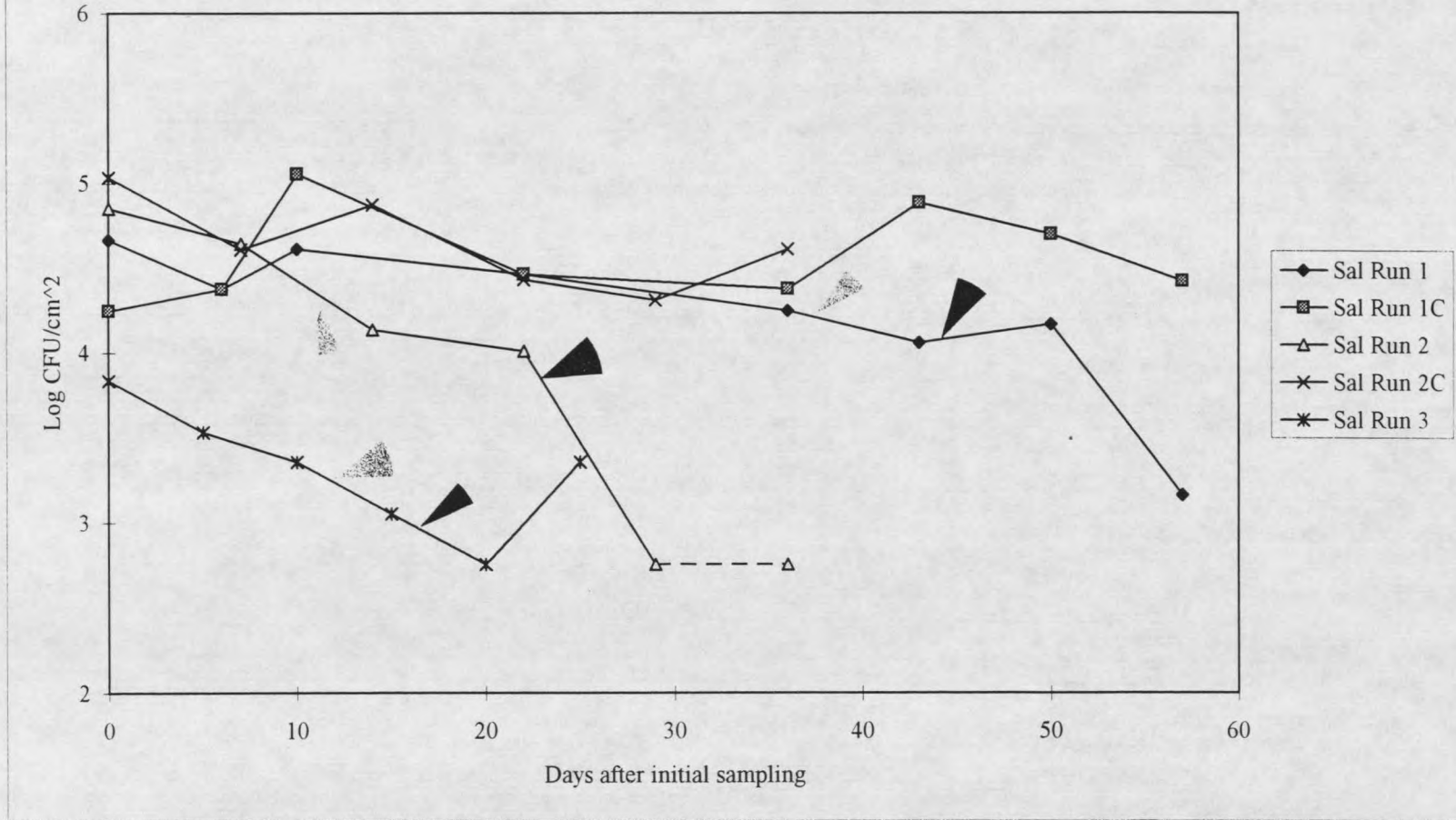
Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (▲). Chlorination at 0.2ppm is indicated by a dark gray wedge (▲).

Figure 23: Salmonella enumeration in bulk phase by FAB



Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▒). Chlorination at 0.2ppm is indicated by a dark gray wedge (◼).

Figure 24: Salmonella enumeration in biofilm by FAB



Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▴).

Salmonella was detected in the bulk phase (Figure 23) and biofilm (Figure 24) through extended periods by fluorescent antibody labelling. The longest experimental run included sampling at Day 57, at which point *Salmonella* cells were still detectable. The actual numbers showed some variability, and the counts were frequently close to the detection limits.

In order to determine if the *Salmonella* were viable or not, a nonselective enrichment was performed with intact biofilm samples from Sal Run 3. *Salmonella* was able to be cultured by this method throughout the experimental run (last sampling point for this procedure was on Day 20), showing at least some of the previously unculturable *Salmonella* cells present in the biofilm were able to be resuscitated under appropriate conditions. Filtered samples with no enrichment placed directly on XLD, and samples that underwent selective enrichment using tetrathionate broth, did not recover *Salmonella* past the Day 7 point.

Results from PCR confirmation agreed with the visual identification of colonies identified as *Salmonella* from XLD. No problems were encountered using this methodology for colony identification. It was found to be generally unnecessary to use PCR as a confirmation method, with none of the heterotrophs from the RO dilution water which were able to grow on XLD resembling *Salmonella* morphologically.

Three of the reactors were treated with chlorine after a period of development (Sal Runs 1, 2 and 3, as shown on Figures 23 and 24). Chlorination at 0.05ppm appeared to have little effect on *Salmonella* numbers. When raised to 0.2ppm chlorine input, *Salmonella* rapidly became undetectable in the reactor bulk phase. *Salmonella* persistence

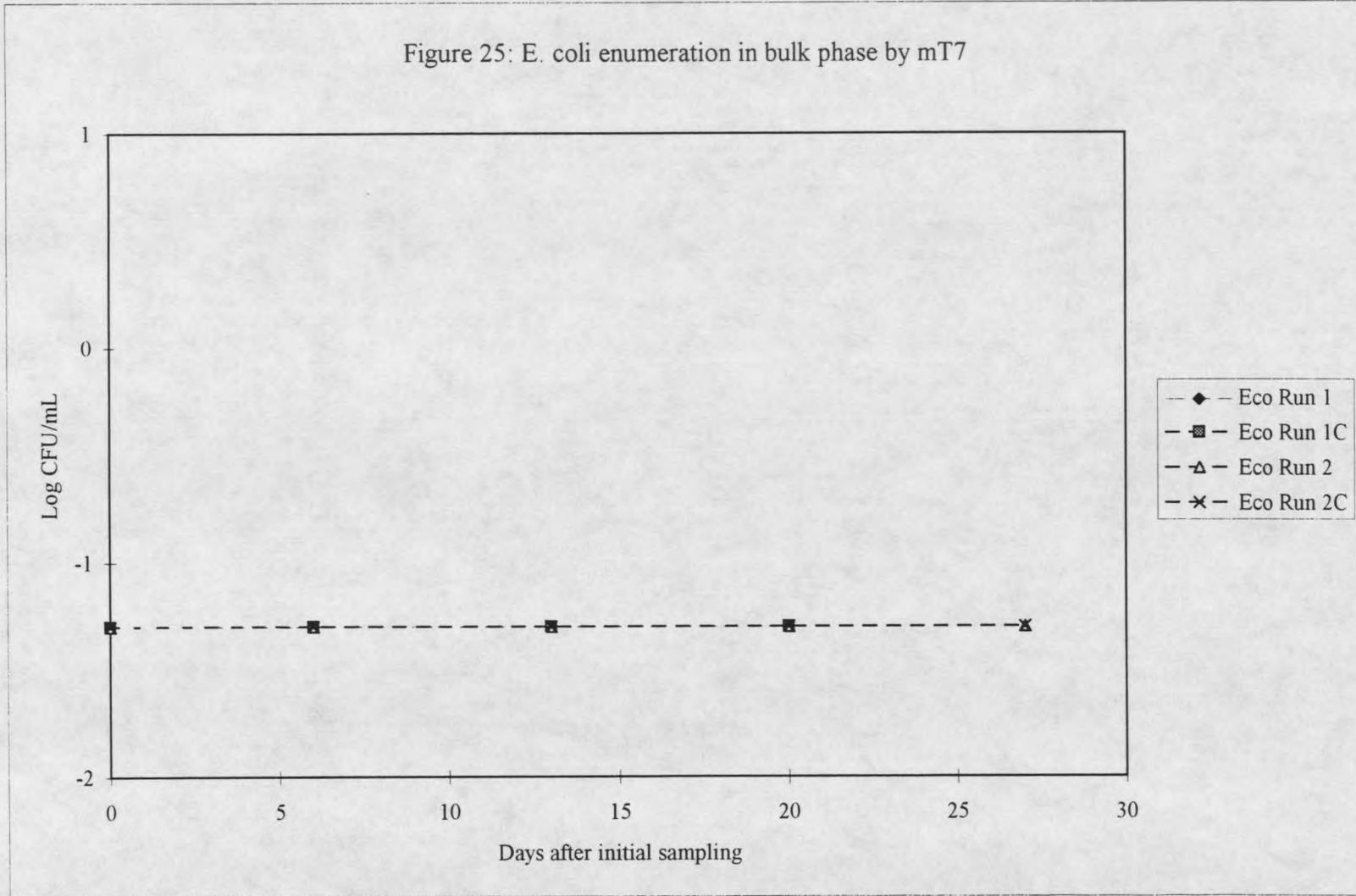
in the biofilm was observed using immunofluorescence following this disinfection level until the end of the experiment in Sal Runs 1 and 3, no persistence was observed in Sal Run 2.

Escherichia coli O157:H7

Two experimental runs with this organism were performed (Eco Runs 1 and 2), along with two concurrent unchlorinated control runs (Eco Runs 1C and 2C). *E. coli* enumeration has been summarized in Figures 25 to 28, with the individual data sets presented in the Appendix.

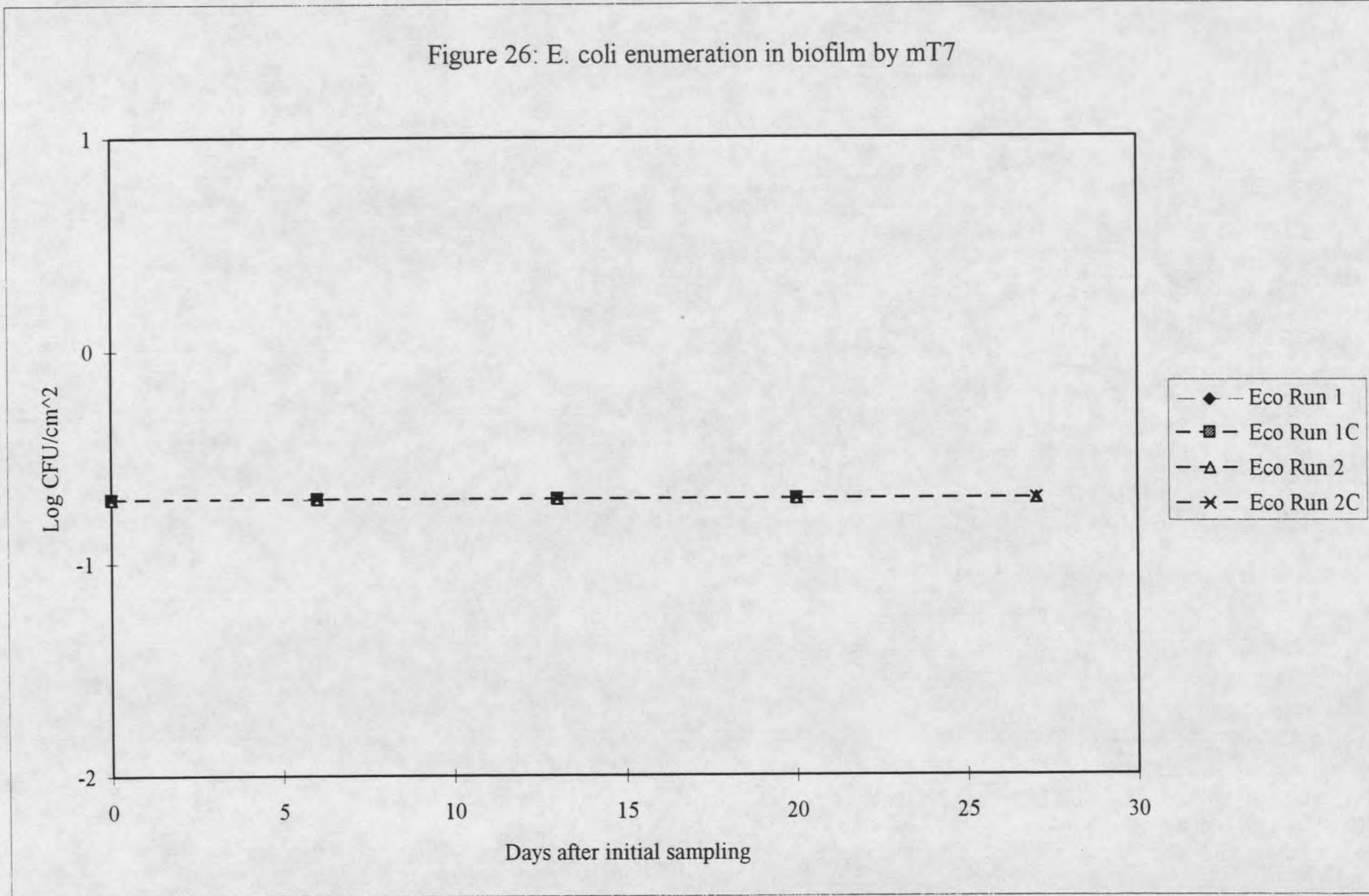
This organism did not generally exhibit persistence in this experimental system. No cells were detected in the bulk phase by either selective plating or antibody staining (Figures 25 and 27), or in biofilm samples using selective plating (Figure 26). Some persistence was observed in the biofilm of Eco Runs 1C and 2C using antibody staining (Figure 28) at levels just above the detection limits for this method. This does not necessarily demonstrate viable cells, and no viability studies were carried out for this organism. No persistence was observed past 2 weeks in the reactors, even using immunofluorescence.

Figure 25: E. coli enumeration in bulk phase by mT7



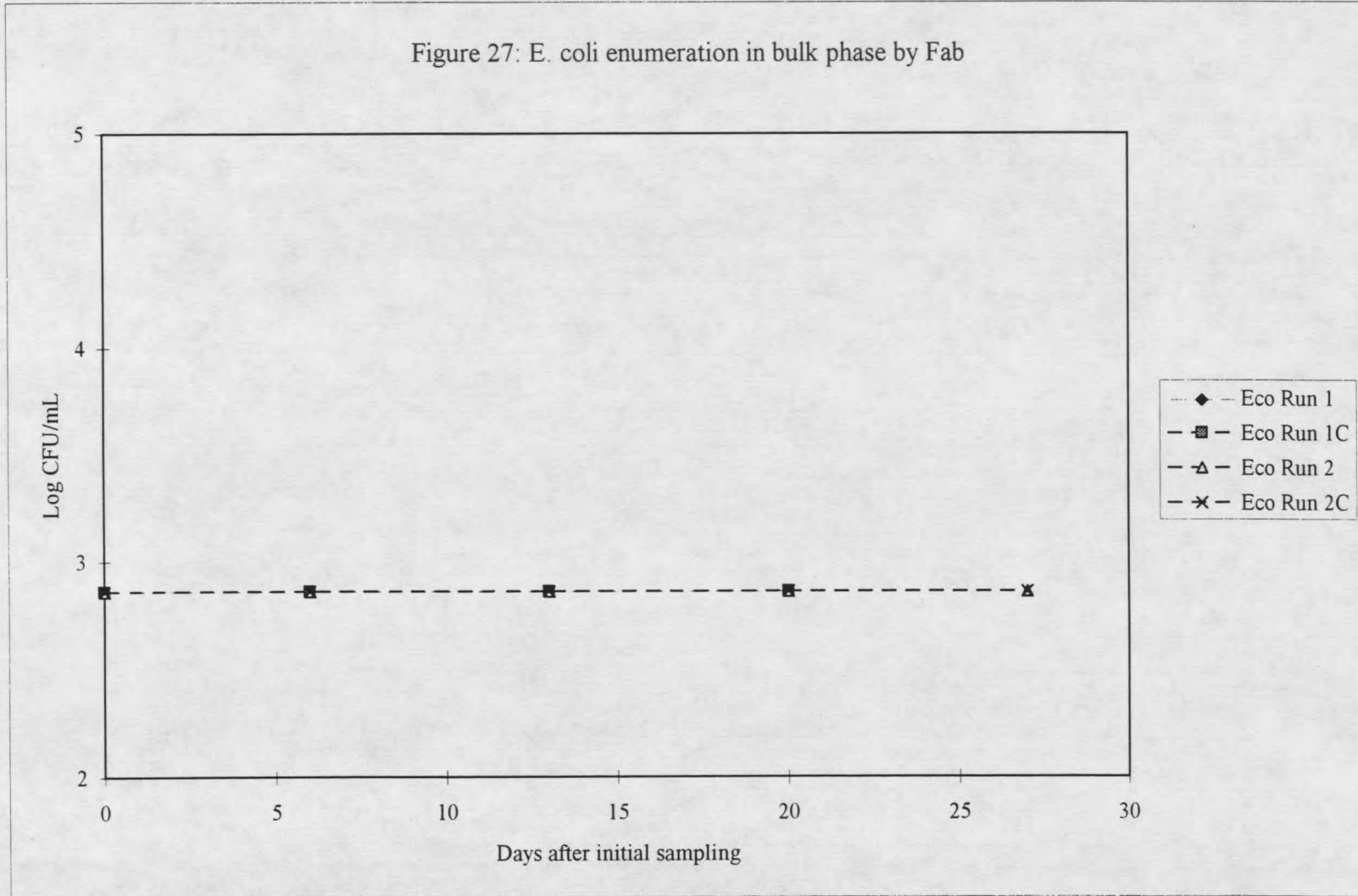
Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▤).

Figure 26: E. coli enumeration in biofilm by mT7



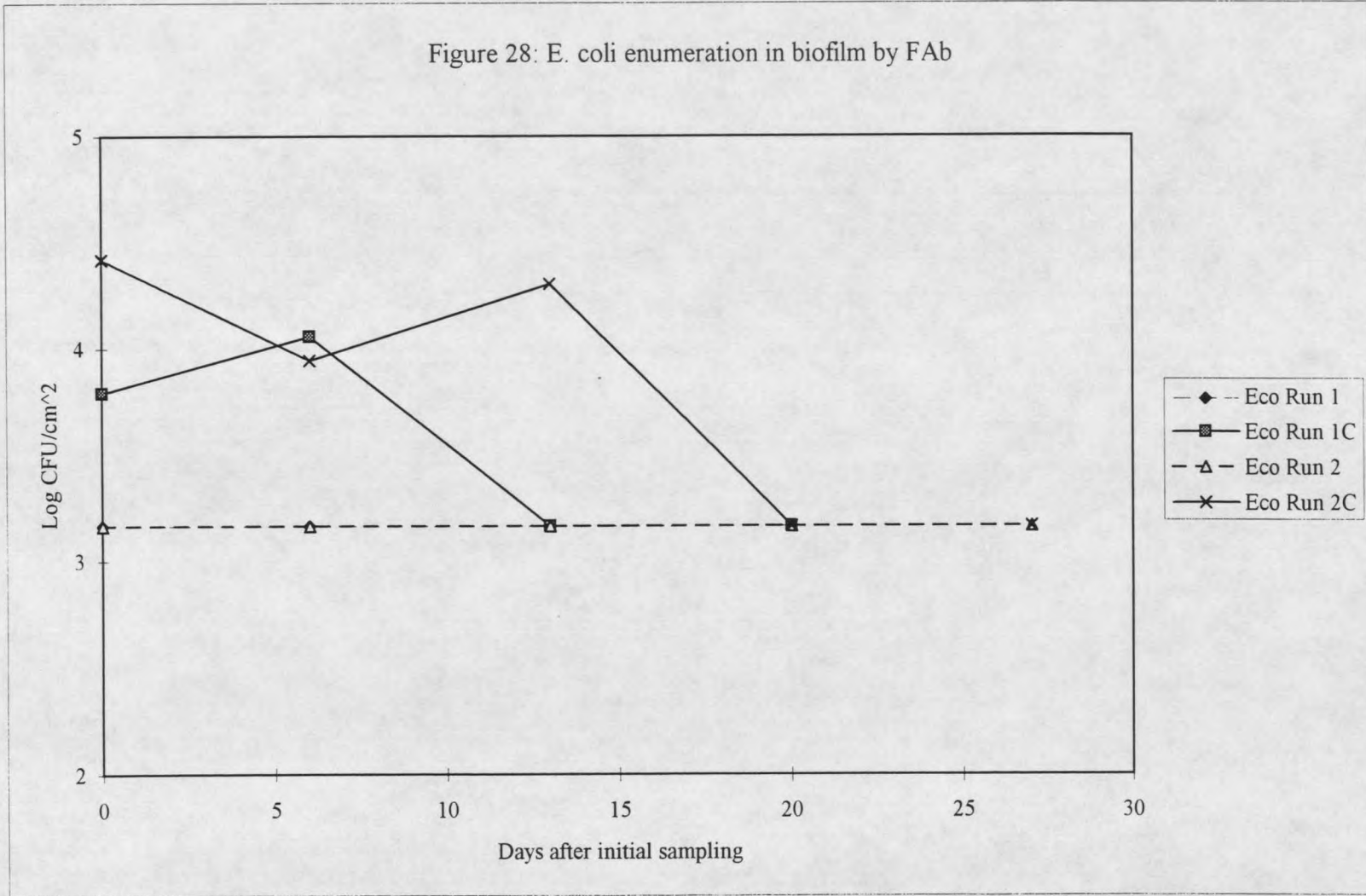
Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (▲). Chlorination at 0.2ppm is indicated by a dark gray wedge (▴).

Figure 27: E. coli enumeration in bulk phase by Fab



Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▴).

Figure 28: E. coli enumeration in biofilm by FAb



Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (▒). Chlorination at 0.2ppm is indicated by a dark gray wedge (▀).

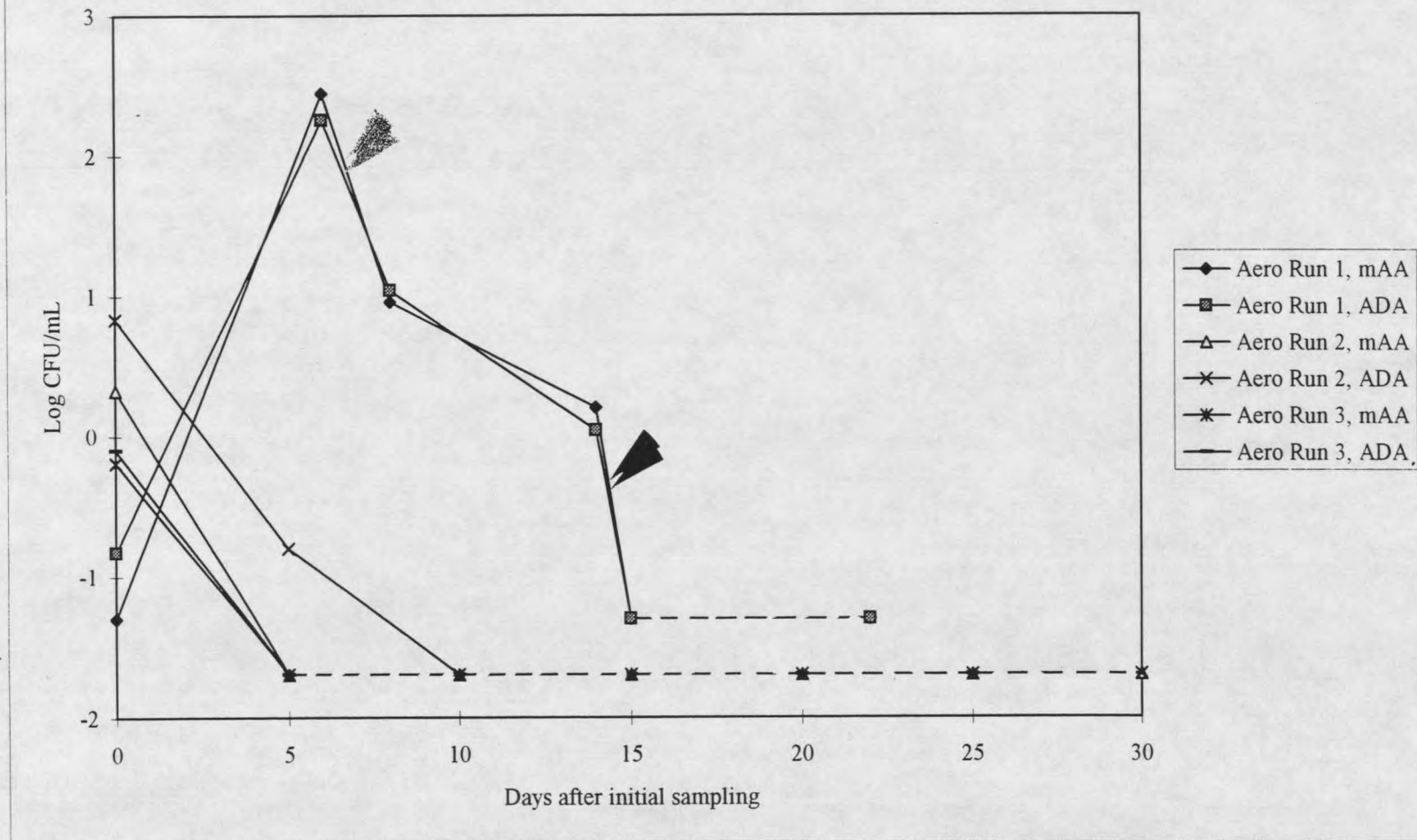
Aeromonas hydrophila

Three runs examining *Aeromonas* were performed (Aero Runs 1, 2 and 3), all of which were chlorinated after a development period. Enumeration data from these runs is presented in Figures 29 to 32, with the individual data sets presented in the Appendix.

From the data gathered from Aero Runs 2 and 3, it would appear that the test *Aeromonas* strains demonstrate a loss of culturability and declining persistence within the bulk phase and biofilm. Colony counts on both m-*Aeromonas* Agar (mAA) and Ampicillin Dextrin Agar (ADA) exhibited a rapid decline from the start of the experiment to a level below detection in both bulk phase (Figure 29) and biofilm (Figure 30). Cells were able to persist, as seen from the fluorescent antibody data (Figures 31 and 32), and undergo a steady decline through the course of the experiment. There did not appear to be appreciable numbers of these organisms in the biofilm. They were detectable for longer periods in the bulk phase. No viability studies were carried out with this organism.

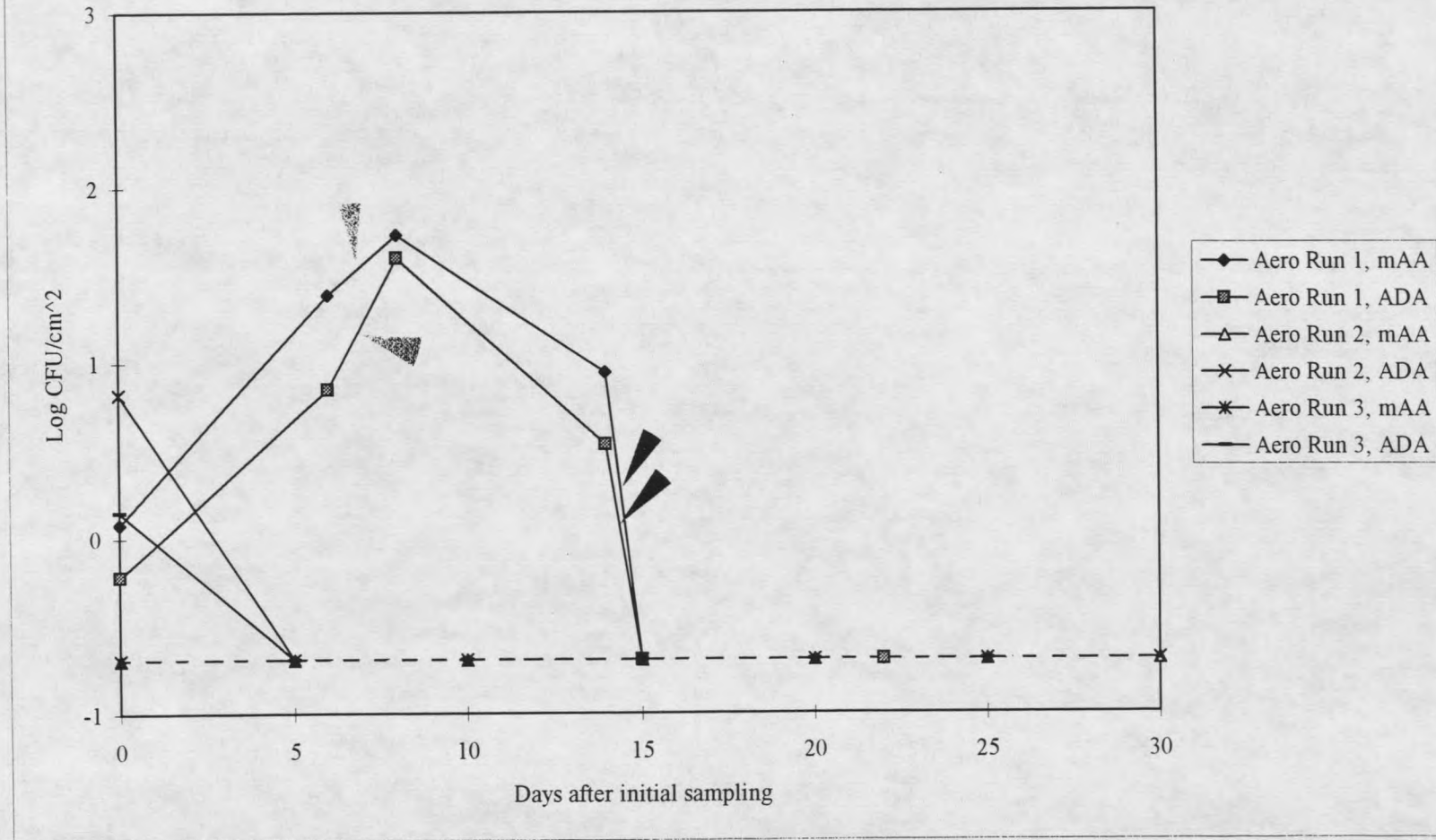
In Aero Run 1, there was an increase in culturable aeromonad counts at Day 6 in both bulk phase (Figure 29) and biofilm (Figure 30). This peak continued through to disinfection at 0.2ppm chlorine. These colonies were confirmed as *A. hydrophila* using PCR. No *A. hydrophila* were detected in the feed water. Whether this increase in aeromonads was due to growth or cell resuscitation was not determined.

Figure 29: Aeromonas in bulk phase by selective media



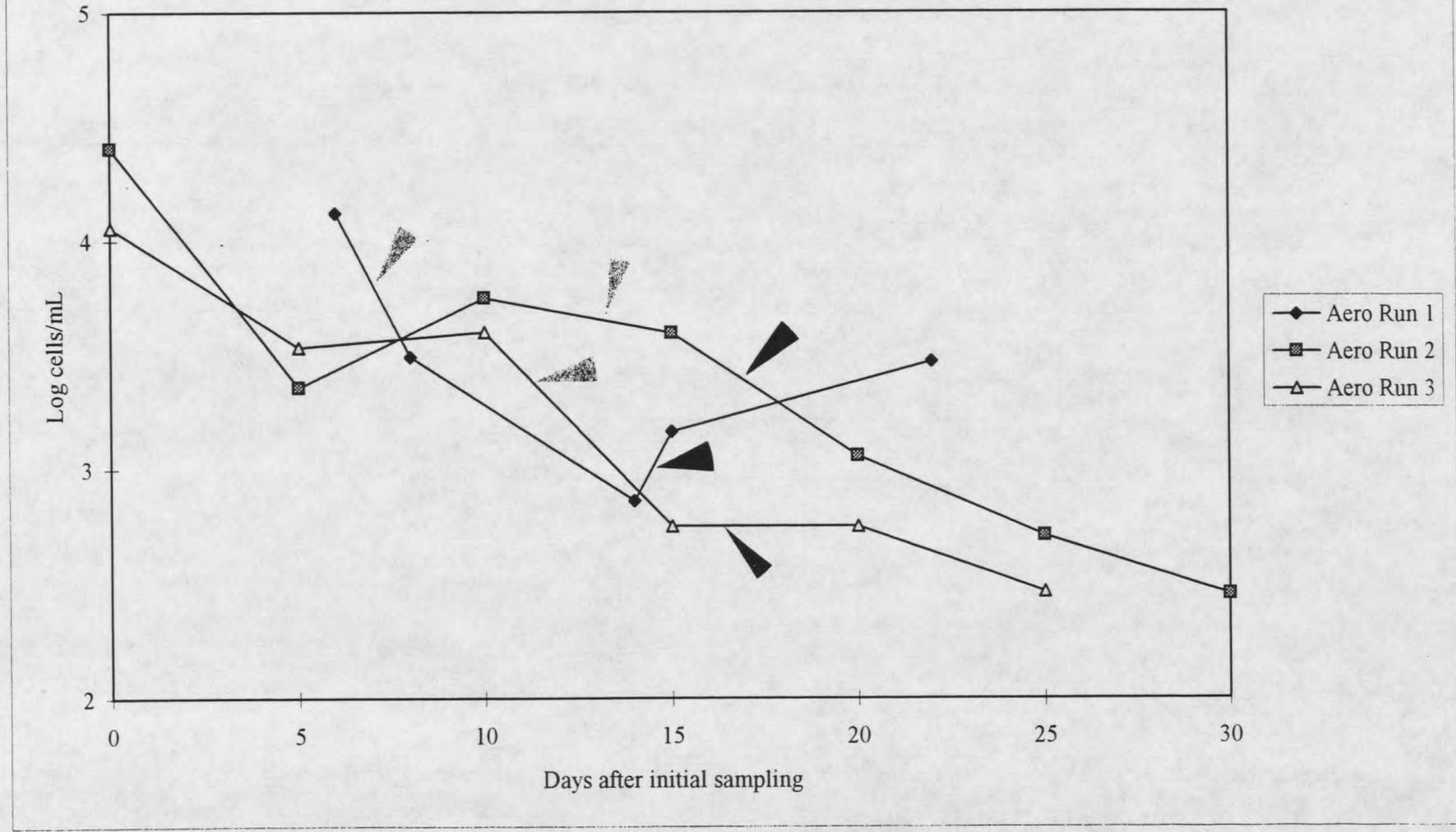
Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▤).

Figure 30: Aeromonas in biofilm by selective media



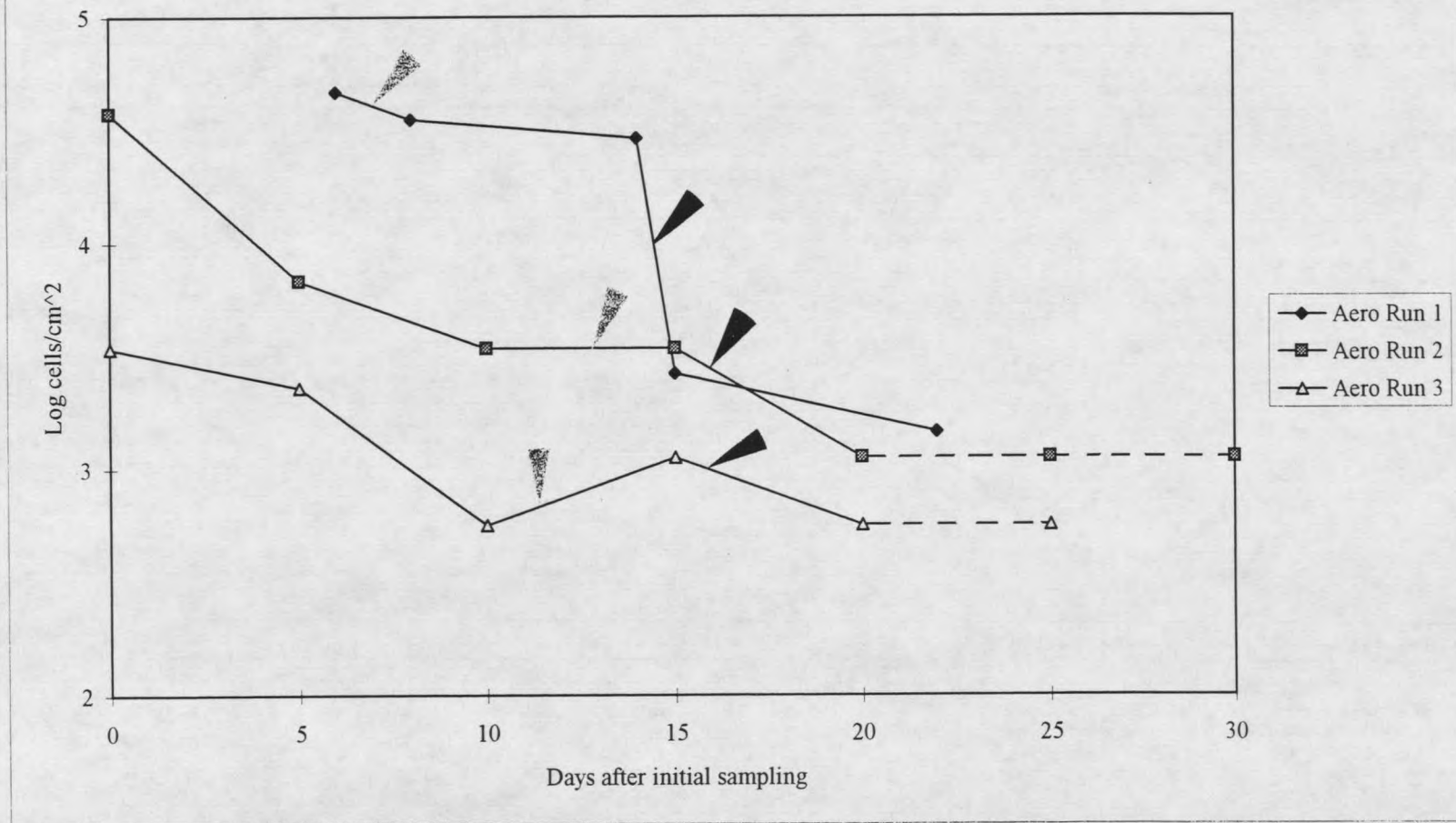
Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▴).



Figure 31: Aeromonas enumeration in bulk phase by FAb



Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (▲). Chlorination at 0.2ppm is indicated by a dark gray wedge (◄).

Figure 32: Aeromonas enumeration in biofilm by FAB



Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (). Chlorination at 0.2ppm is indicated by a dark gray wedge ().

PCR was demonstrated as a useful identification tool here. Colonies isolated from Aero Run 1 on both mAA and ADA included several which were identified as non-aeromonad. Although these colonies were visually different from those identified as aeromonads, they were still similar enough to be potentially identified as aeromonads if not confirmed. These heterotrophs were also isolated from the feed water.

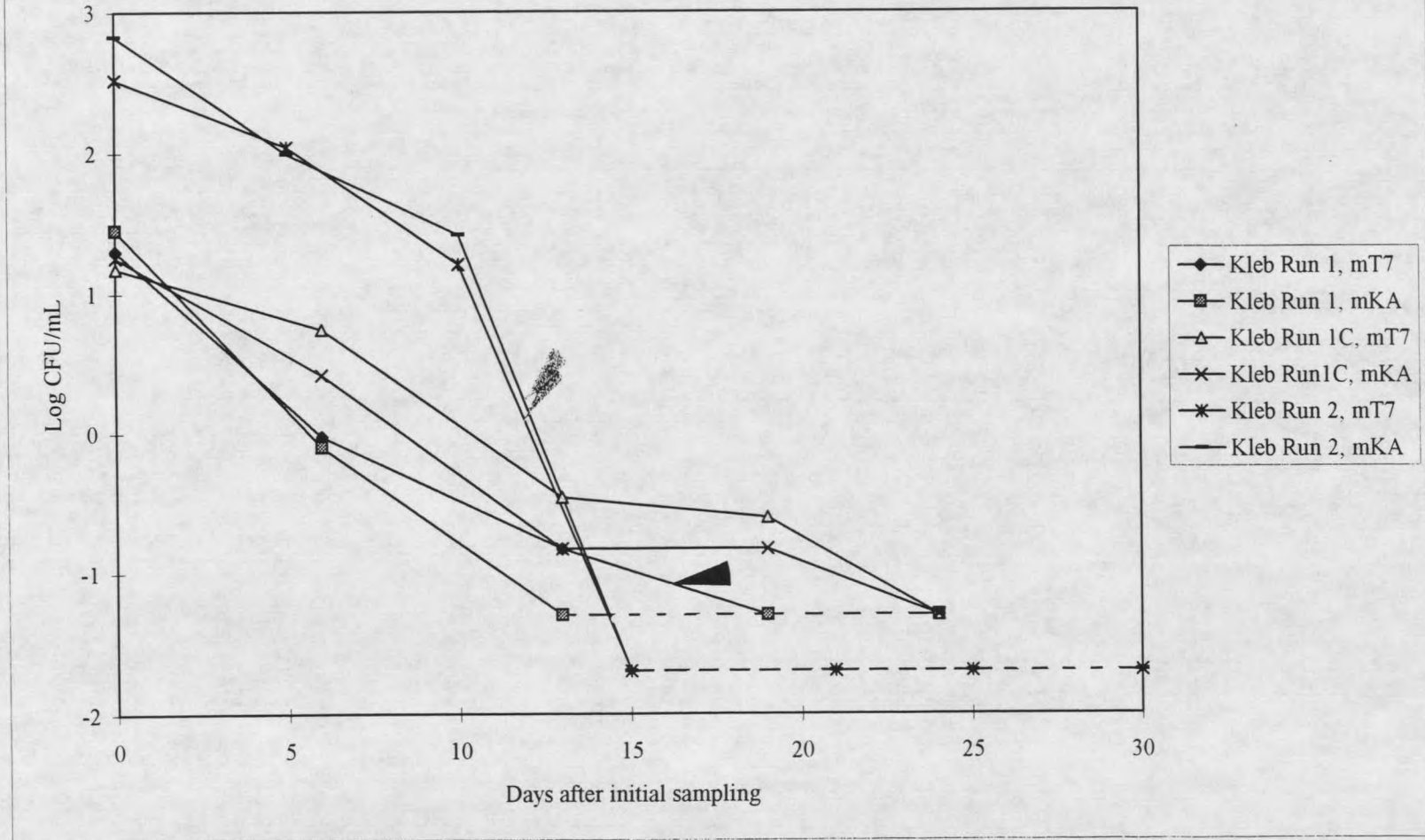
Chlorination appeared to adversely affect the aeromonad population. With 0.2ppm chlorine input, aeromonads were not subsequently detected in biofilms even by immunofluorescence (Figure 32). Numbers in the bulk phase were slightly lower following 0.2ppm chlorination in Aero Runs 2 and 3, and slightly elevated in Aero Run 1 (Figure 31).

There was little observed difference between mAA and ADA for the enumeration of *Aeromonas* under the tested conditions.

Klebsiella pneumoniae

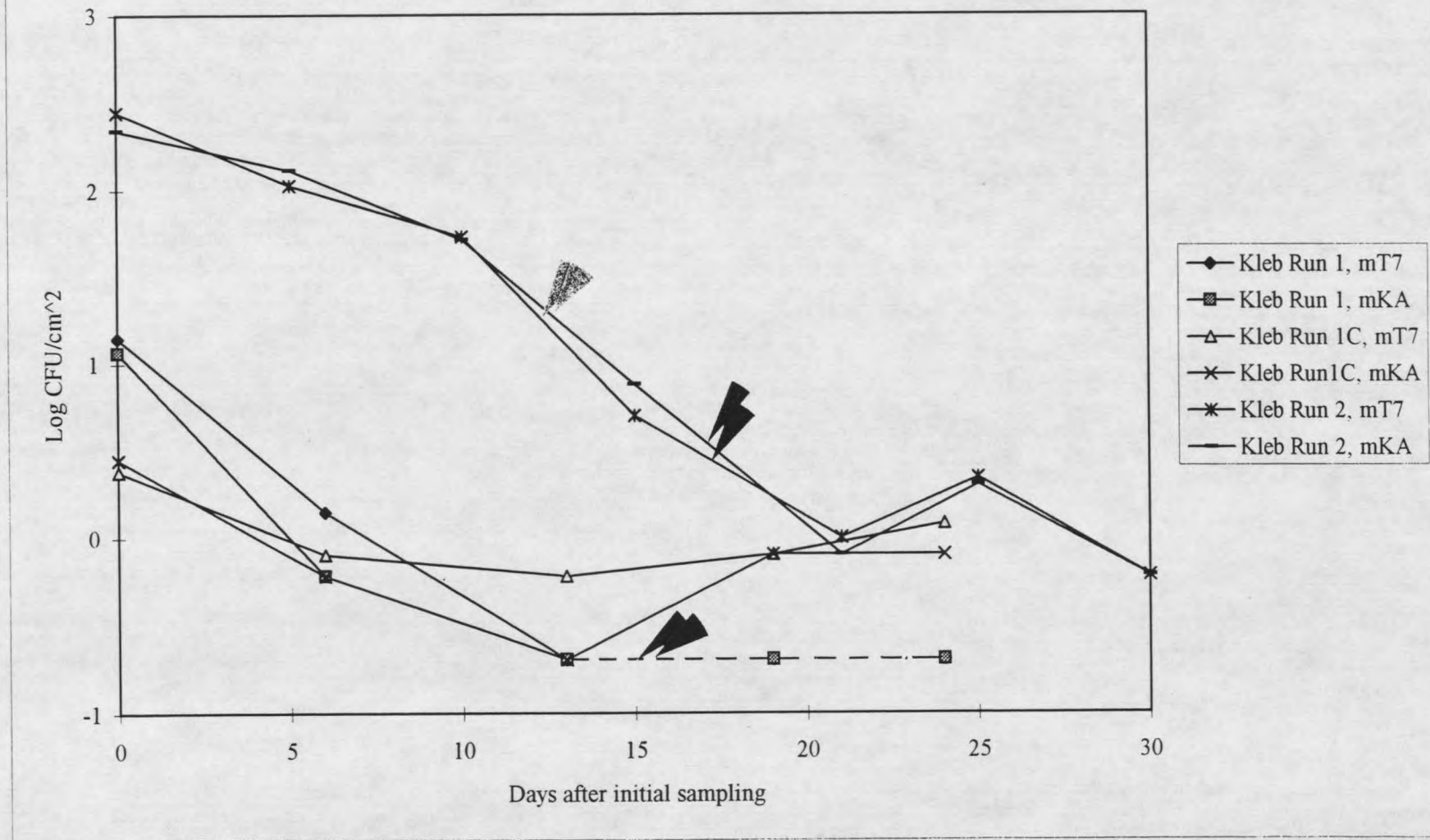
Two experimental runs with *Klebsiella* were performed (Kleb Runs 1 and 2), as well as one unchlorinated control run (Kleb Run 1C) concurrent with Kleb Run 1. *Klebsiella* data gathered in these runs has been summarized in Figures 33 to 36, with the individual run data presented in the Appendix.

Figure 33: Klebsiella in bulk phase by selective media



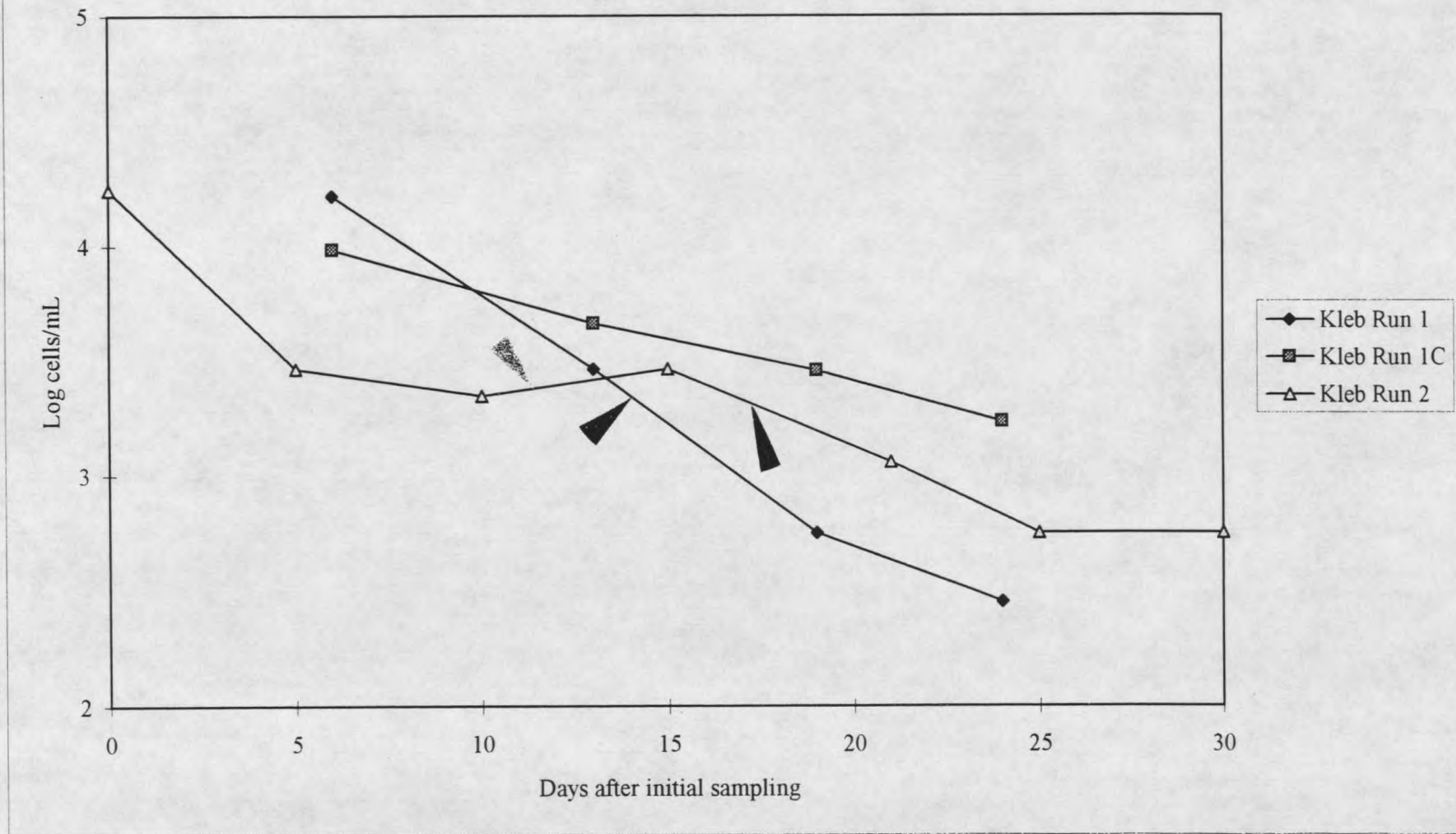
Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▴).

Figure 34: Klebsiella in biofilm by selective media



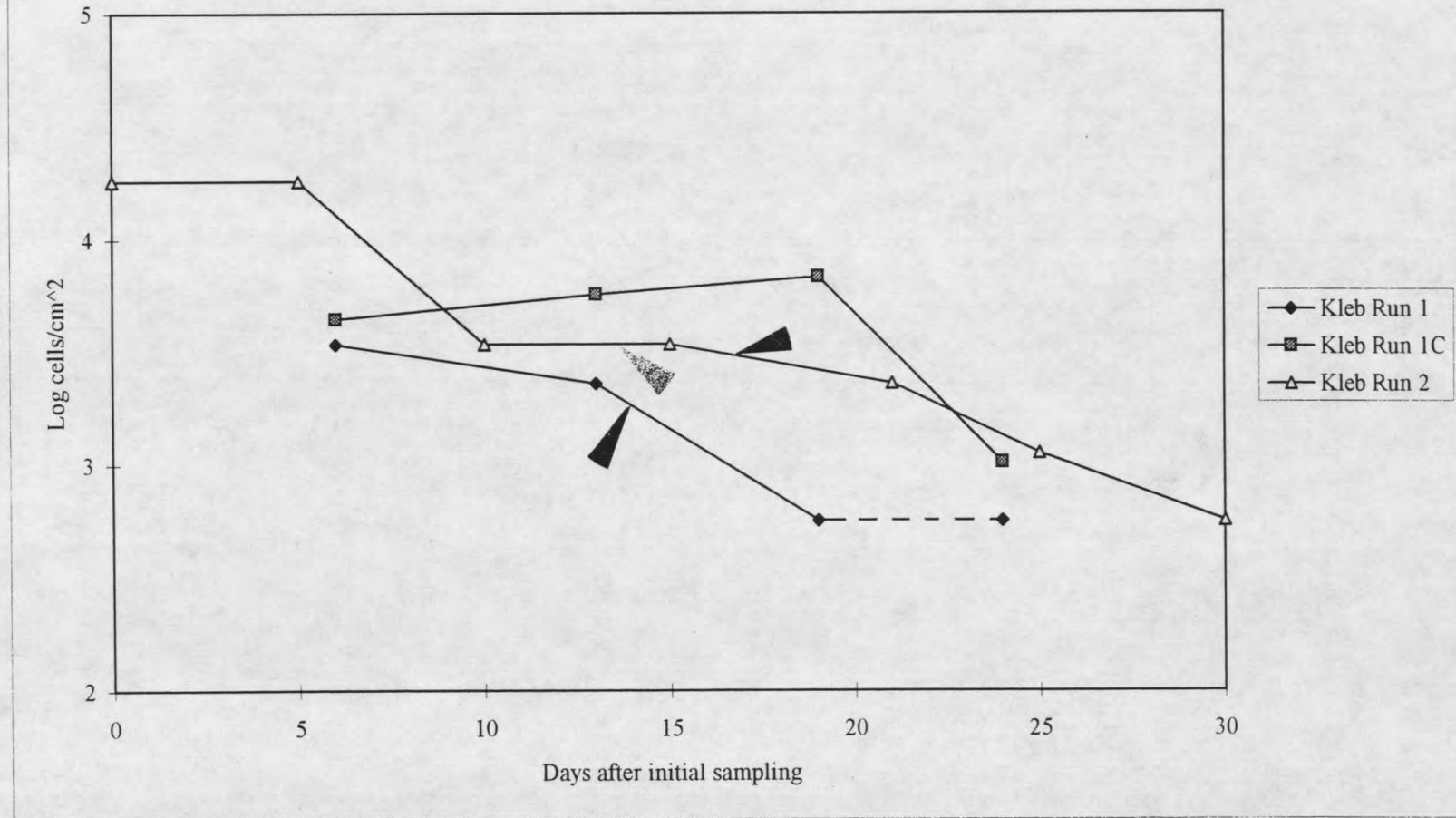
Horizontal dotted line indicates data is below detection level, result of <n presented as 0.5n. Chlorination at 0.05ppm is indicated by a light gray wedge (◄). Chlorination at 0.2ppm is indicated by a dark gray wedge (◄).

Figure 35: Klebsiella enumeration in bulk phase by FAb



Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▤).

Figure 36: Klebsiella enumeration in biofilm by FAb



Horizontal dotted line indicates data is below detection level, result of $<n$ presented as $0.5n$. Chlorination at 0.05ppm is indicated by a light gray wedge (▨). Chlorination at 0.2ppm is indicated by a dark gray wedge (▴).

Kleb Runs 1 and 1C show some persistence of the organism in the reactor.

Culturability steadily declined to a non-detectable level by the end of the experiments in the bulk phase (Figure 33), and declined in a variable fashion in the biofilm (Figure 34). Cells were detectable in slowly declining numbers by immunofluorescence in the bulk phase (Figure 35) and biofilm (Figure 36).

With the data from Kleb Run 2, this organism appears to be well able to colonize and persist in the experimental system. Selective plate counts are lower than the fluorescent antibody counts by 1 to 2 logs in both bulk phase (Figures 33 and 35) and biofilm (Figures 34 and 36) prior to chlorination, as opposed to 3 logs or more as was the case with the other organisms examined. There appeared to be a slow overall decline in total klebsiellae numbers over the course of the experiment in both bulk phase and biofilm, as measured by immunofluorescence (Figures 35 and 36). Chlorination at 0.05ppm resulted in culturable klebsiellae numbers dropping to below detection level in the bulk phase (Figure 33), but they were still culturable from the biofilm up until the last experimental point (Figure 34).

There was little observable difference between mT7 agar and m-Klebsiella agar in enumeration of *Klebsiella* under the tested conditions.

Disinfection effects upon test organisms

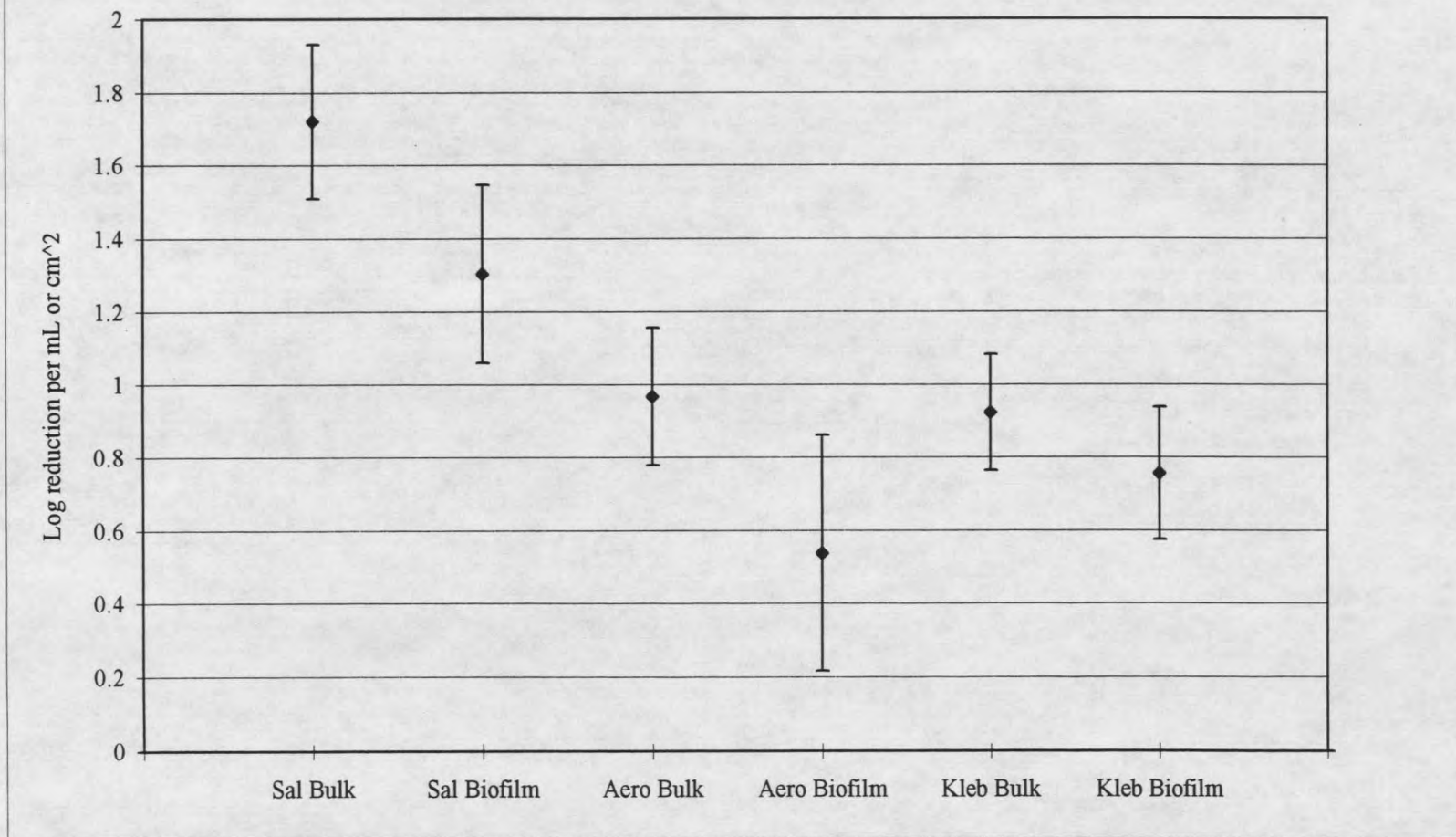
The effects of chlorine in the reactors upon the test organisms have been summarized in Figure 37. This has been performed in a similar fashion to the analysis of

chlorination effects on heterotrophs (as shown in Figures 19 and 20), although only the immunofluorescence data were used since culturable counts were typically below detection. The data involving *E. coli* O157:H7 are not shown, as this organism was frequently not detectable even by immunofluorescence at the stage when chlorination was begun. Two data sets were examined, composed of the logarithm of the raw values from unchlorinated reactors, and from those receiving 0.2ppm chlorine. The effect of 0.05ppm chlorination is not examined, since too few data points were gathered to allow comparison. Influence of chlorine is shown by log reduction of counts between unchlorinated and chlorinated data sets.

The purpose of Figure 37 is to demonstrate that 0.2ppm chlorination did reduce the numbers of the target organisms in the reactors, and that this reduction was more apparent in the bulk phase than in the biofilm. In comparison between organisms, it should be remembered that counts were not always at a steady level before chlorination, and were frequently already in a slow decline. Also, the amount of reduction of cell counts is limited by the detection level. Many of the pre-chlorination counts were close to this level, and many of the post-chlorination counts were below it. This makes quantitative evaluation of chlorination effects almost impossible.

With these considerations in mind, it should be noted that *Salmonella* cell numbers were heavily reduced following chlorination, particularly in the bulk phase. *Aeromonas* and *Klebsiella* numbers were reduced somewhat, although it is unclear whether this was due to chlorination or the steady decline of numbers already noted.

Figure 37: Effects of 0.2ppm chlorine input on immunofluorescence counts of test organisms.



Log reduction calculated by subtraction of mean of log chlorinated values from mean of log unchlorinated values.
Error bars represent the square root of the sum of squares of standard error from chlorinated and unchlorinated data sets.

Examination of intact biofilms using microscopy

In order to gain further information about the behaviour of the test organisms in experimental biofilms, intact biofilm samples were taken and stained using the propidium iodide - antibody double staining method. These slides were then examined using the confocal microscope. This was not intended to yield quantitative data, but instead give information on spatial patterns of the test organisms, such as forming clusters or microcolonies or remaining as single cells. Also of interest were spatial relationships to the general heterotrophic population.

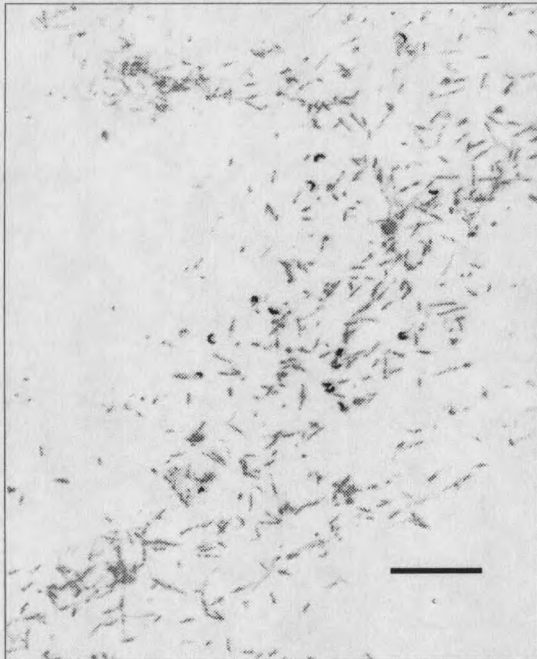
In all cases the biofilm was relatively thin, and remained a patchy monolayer of cells through the experiment. This is apparent in the following frontal images of intact biofilms. Distribution of cells was quite heterogeneous on the slides.

The images shown are representative examples. The images have been gray-scaled and had pixel signal reversed from the original color merges. In some of the images the target cells appear larger than the surrounding heterotrophs, this is due to the antibody staining being somewhat more diffuse than the propidium iodide staining, and thus surrounding the cell.

Salmonella typhimurium

Images of the biofilm without chlorination at Week 2 (Figure 38) and Week 6 (Figure 39) are shown here. As has been noted, this organism demonstrated a capacity to persist in the biofilm through the unchlorinated stages of the experiments. This persistence

Figure 38: *Salmonella* RT biofilm, unchlorinated, week 2.



84
Figure 39: *Salmonella* RT biofilm, unchlorinated, week 6.

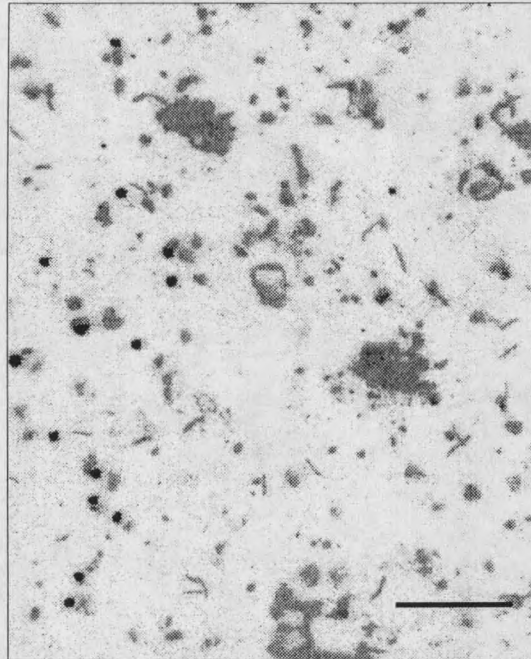


Figure 40: *Salmonella* RT biofilm after 0.05ppm chlorination, week 3.

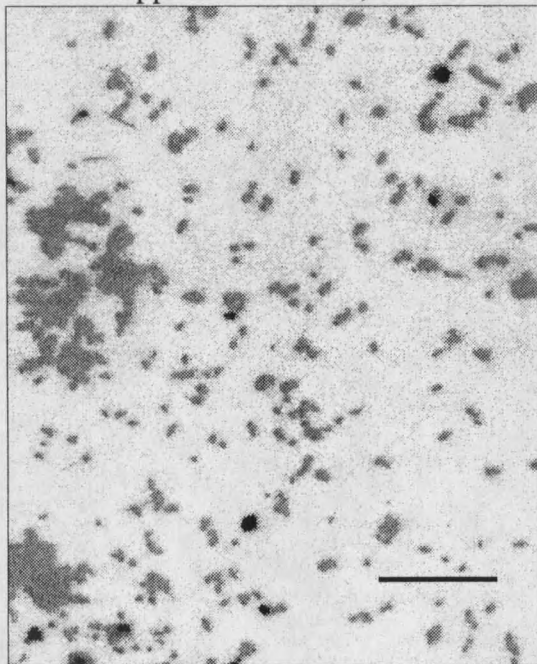
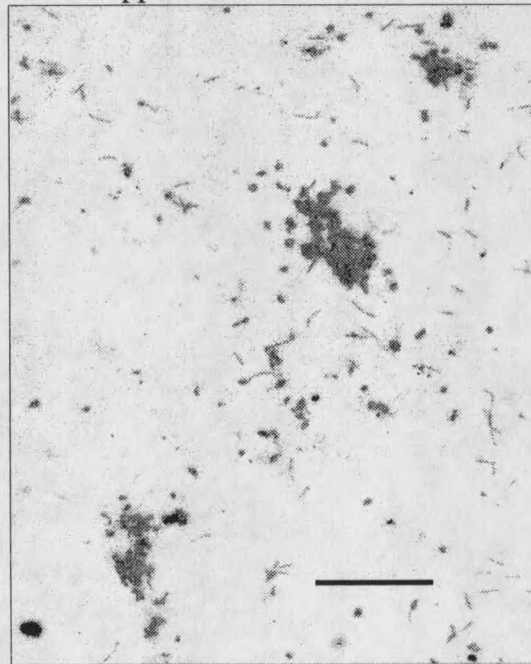


Figure 41: *Salmonella* RT biofilm after 0.2ppm chlorination, week 5.



Bar indicates 25 μ m.

Target organisms stained by immunofluorescence appears black.

Other organisms stained only with propidium iodide appear gray.

appeared to be in the form of single cells scattered on the substrata. These cells gradually becoming nonculturable while still (at least with some cells) remaining viable. No small groups (microcolonies) of these cells were observed.

Dosing at 0.05ppm chlorine on a Week 3 biofilm had little effect (Figure 40). When this was increased to 0.2ppm *Salmonella* numbers were reduced, but were still detectable in 2 of the 3 *Salmonella* runs (Figure 41).

Following the nonselective enrichment procedure, images were also taken of the biofilm (Figure 42). The *Salmonella* do not appear to be forming microcolonies in the biofilm even after this treatment. There are several heterotrophic microcolonies visible.

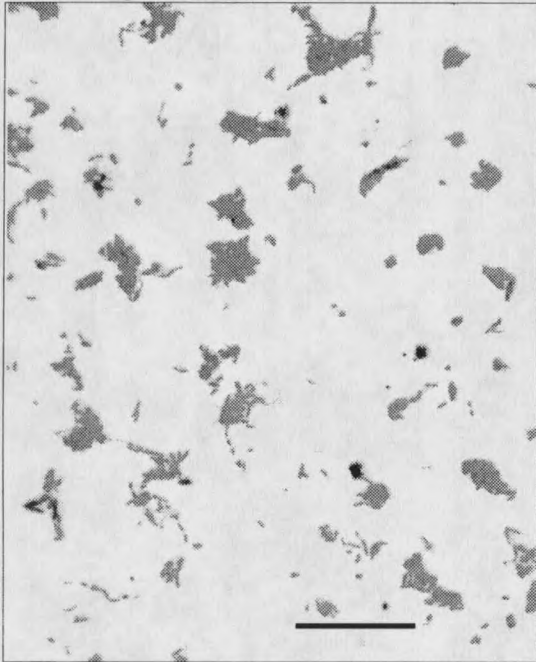
Escherichia coli O157:H7

As already noted, this organism did not appear to persist well in the biofilm. One or two scattered cells were seen in some early samples, but images were more usually found to contain heterotrophic bacterial cells only. Figure 43, a Week 1 image from Eco Run 2, is typical in this respect.

Aeromonas hydrophila

Aeromonads were found to persist somewhat in the biofilm, usually as single cells. Images from Week 1 (Figure 44) and Week 2 (Figure 45) of Aero Run 3 are shown here. Following chlorination (Figure 46), these cells were usually not seen in the biofilm.

Figure 42: *Salmonella* RT biofilm following nonselective enrichment.



86 Figure 43: *E. coli* RT biofilm, unchlorinated, week 1.

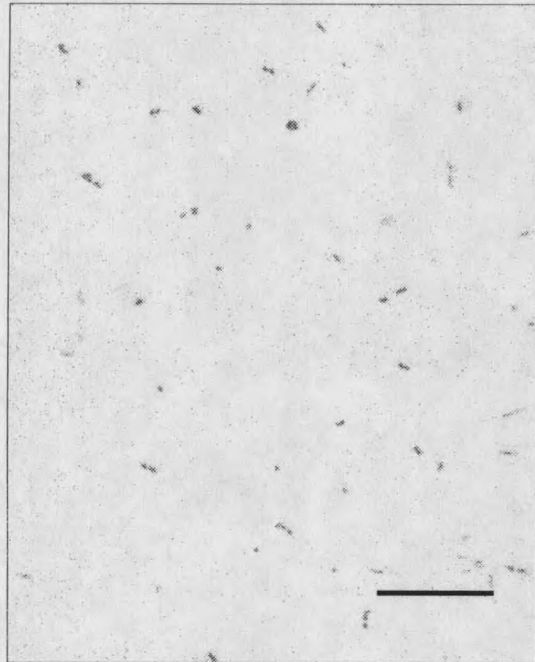


Figure 44: *Aeromonas* RT biofilm, unchlorinated, week 1.

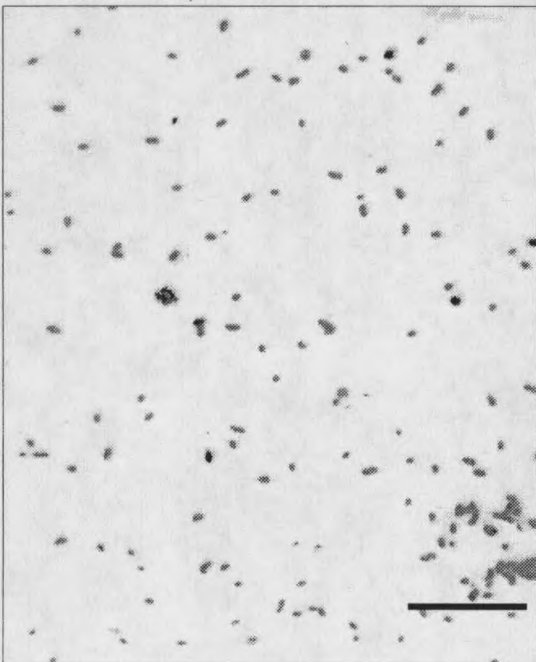


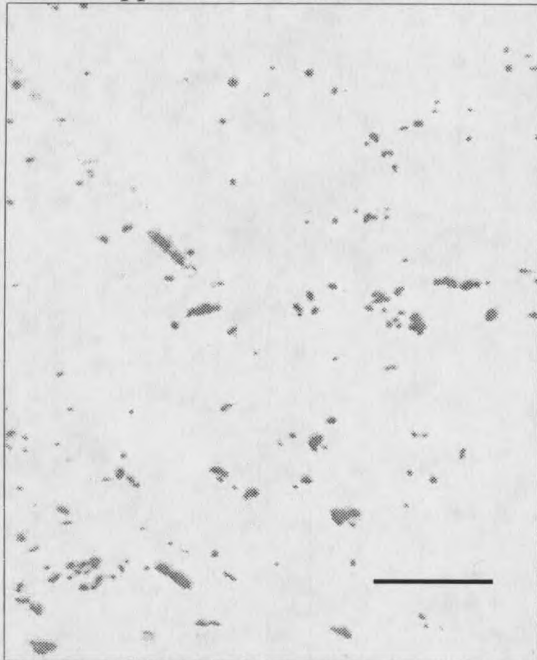
Figure 45: *Aeromonas* RT biofilm, unchlorinated, week 2.



Bar indicates 25 μ m.

Target organisms stained by immunofluorescence appears black.
Other organisms stained only with propidium iodide appear gray.

Figure 46: *Aeromonas* RT biofilm after 0.2ppm chlorination, week 4.



87

Figure 47: *Klebsiella* RT biofilm, unchlorinated, week 2.

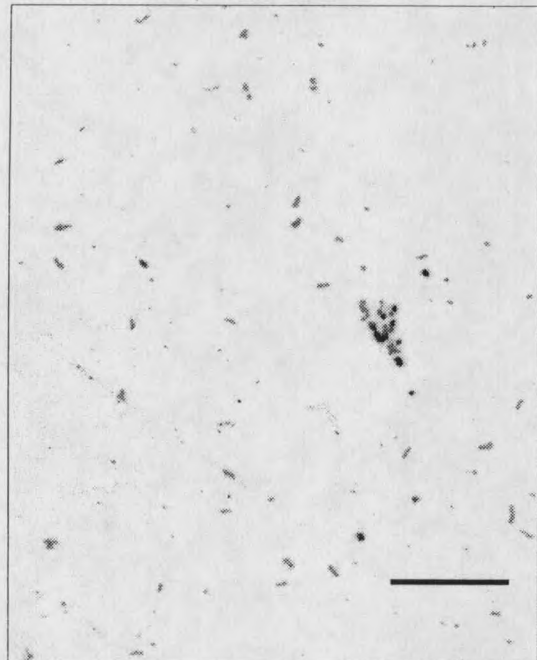


Figure 48: *Klebsiella* RT biofilm, unchlorinated, week 2.

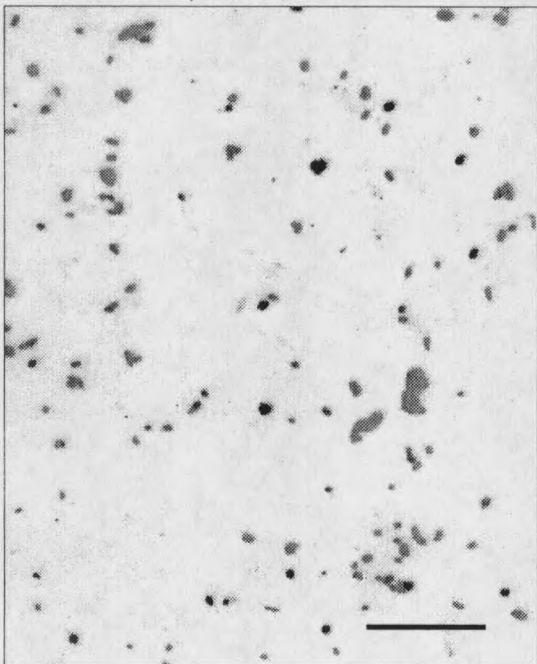


Figure 49: *Klebsiella* RT biofilm after 0.2ppm chlorination, week 4.



Bar indicates 25 μ m.

Target organisms stained by immunofluorescence appears black.
Other organisms stained only with propidium iodide appear gray.

Klebsiella pneumoniae

Different and irregular spatial distributions of *Klebsiella* were observed in intact biofilm samples. Cells formed small clusters (Figure 47) and were also seen as single cells (Figure 48) within the first 2 weeks of the experiments. Formation of clusters or microcolonies would be expected for this organism, given its lack of motility and its known capacity to colonize the substrata under drinking water conditions. However, no quantitative increase was observed with selective media or immunofluorescence of *Klebsiella* in this study (Figures 33 to 36). This is reinforced by qualitative observation with immunofluorescence of the intact biofilm, in which large formations of the organism were not detected. Following chlorination at 0.2ppm, *Klebsiella* numbers appeared to decline, although not appreciably faster than before chlorination. Figure 49 shows biofilm from Kleb Run 2 after a week of disinfection at 0.2ppm chlorine input. A few *Klebsiella* cells are still apparent in this image.

Heterotrophic population

As can be seen from Figures 38 to 49, the heterotrophic population showed variable behaviour in forming a biofilm. Where there was little crowding, cells tended to be scattered and single, while in more crowded situation after development of the biofilm (such as Figures 39 and 40) saw the development of microcolonies. These were also observed following nonspecific enrichment (Figure 42). There was no obvious interaction between the test organisms and the heterotrophs, such as formation of mixed clusters.

DISCUSSION

The intended scope of this study was to determine if selected bacteria would persist within a biofilm under drinking water conditions, as simulated and observed in a laboratory-scale reactor. The described results have several implications when applied to a macro-scale environment, such as an actual drinking water distribution system. While these environments have such variation that no test system could be described as "typical", observations of bacterial behaviour from the test system in these experiments can be generalized regarding what may be expected from the tested organisms.

Use of coliforms to predict the behaviour of pathogens

The examined organisms showed some similarities in behaviour under the test conditions, but also some important differences. For example, amongst the known pathogenic organisms examined, *Salmonella typhimurium* showed a long term persistence within the biofilm, with maintenance of viability amongst some cells, whereas *Escherichia coli* O157:H7 did not appear to colonize the substrata well, and was usually washed out of the reactor by the time sampling had begun. These organisms are known to be quite closely related taxonomically, yet appear to behave quite differently in this tested environment. As noted previously, coliform bacteria are usually the sole measure employed to evaluate water for the contamination of pathogenic organisms, therefore taken beyond their original role of indicating recent fecal pollution. Given the behavioural

differences observed here between these two members of the same taxonomic family, the utility of coliforms to predict the long-term survival of a variety of pathogens within a distribution system would seem to be quite limited.

Klebsiella pneumoniae was examined in this study as a representative coliform, known to be often present and capable of regrowth within a distribution system (Geldreich and Rice, 1987). While growth of *Klebsiella* was not well demonstrated in this study, its behaviour was dissimilar to both *S. typhimurium* and *E. coli* in that it persisted better and remained culturable longer than these organisms in biofilms. *Klebsiella* could not be said to be predictive of the pathogens in this environment.

Implications of observed physiological changes

The physiological changes observed in cells under tested conditions are of some note. All of the tested organisms showed a reduction in colony counts using enumeration on selective media over time. Several of the organisms did show continued persistence as determined by immunofluorescence, the difference between methods being attributable to injury, starvation, transformation to a viable but nonculturable state, and cell death. This illustrates the limitations of using selective media to detect and enumerate cells following environmental stresses. While less selective media will detect some of the stressed population, as time progresses an increasing proportion of the target organisms will become undetectable even by these methods. Given the complete nonculturability observed in long term persistence of the tested bacteria, including nonculturability

following selective enrichment in some cases, detection by selective media of such cells in an actual distribution system would appear quite unlikely. As some cells were shown to be still viable, their detection would be desirable. The recent advances in molecular detection methods appear to be the most likely solution to detection problems, although the numerous difficulties in actually applying these methods routinely to real situations currently precludes their usage, and in many cases do not give information about the viability of detected organisms.

Effects of surfaces

The role of surfaces as a reservoir of cells within a system has also been shown by this study. Conditions were such that maintenance of target cell numbers within the bulk phase would have involved some growth of or release of cells attached to the substrata. In the absence of disinfection, this appeared to be the case with those bacteria used in this study which did persist in the reactors. Attachment as a mechanism to escape disinfection was also demonstrated, with attached cell numbers appearing more resistant to chlorination than those in the bulk phase. This agrees with resistance to disinfection of attached cells seen in other studies (Herson et al, 1987; LeChevallier et al, 1988). These surface persistence and survival effects would be likely increased under real conditions, with a more developed biofilm, rougher substrata, and likely higher disinfectant levels as well as lower nutrient levels (Huck et al, 1991; Kaplan et al, 1994) through much of a distribution system.

Behaviour of different organisms

There is, to date, very little published information available about the behaviour of specific pathogenic organisms within a drinking water distribution system. Such information is usually concerning behaviour within the bulk phase of the system, rather than interacting with surfaces such as sediments or biofilms. As demonstrated by this and other studies previously mentioned, these interactions are essential for the persistence and survival of these organisms in such a system. As also demonstrated here, prediction of the behaviour of organisms has many difficulties, even in a relatively well defined environment such as a laboratory annular reactor.

Heterotrophic population

The heterotrophic population behaved in the same fashion between experimental runs, indicating a similar growth environment. The population in the bulk phase stabilized to a relatively constant level a few days after the introduction of the heterotrophic population. This is expected due to the constant flow of these organisms through the reactor. Biofilm numbers of heterotrophs varied over time more than the bulk phase, probably due to the initial development of the biofilm. Disinfection at 0.05ppm chlorine input had virtually no effect on the heterotrophic population. Disinfection at 0.2ppm significantly reduced culturability, although it had little effect on viability or total cell numbers. Cells in the bulk phase were more affected by this disinfection than those in the biofilm. Culturability of bulk phase cells following 0.2ppm chlorination, determined by 2

day R2A incubation, dropped much more than with 7 day incubation. This was not observed with biofilm cells, where there was a similar reduction with both methods.

These results agree with the findings of previous reports (McFeters, 1990; Yu and McFeters, 1994) of a reduction in culturability but not viability following chlorination at low (around 0.25ppm) levels. This can lead to overestimation of disinfection efficacy if only plate counts are used to assess numbers of heterotrophic bacteria.

Salmonella typhimurium

The behaviour of *Salmonella* under experimental conditions agrees with observations from other studies. Colonization of and persistence on surfaces was observed by Camper, et al (1985) on activated carbon under drinking water conditions. Viable but nonculturable cell formation with *Salmonella enteritidis* was observed in river water microcosms by Roszak, et al (1983). In the descriptions of the Gideon, MO (Clark et al, 1996), and Riverside, CA (Boring et al, 1971; A collaborative report, 1971) outbreaks of salmonellosis it was speculated that a release of surface-associated *Salmonella* were possibly responsible for the waterborne outbreaks. The experimental data shown here would support such a hypothesis, showing long-term survival and surface-association of the Gideon outbreak strain of *S. typhimurium*, particularly in unchlorinated waters.

This organism demonstrated a greater chlorine sensitivity than *Aeromonas* or *Klebsiella*. The waterborne outbreaks noted above both occurred in unchlorinated systems. Water with no chlorine residual, even if originating from a high-quality source, remains a public health liability due to potential contamination during distribution.

Escherichia coli O157:H7

The comparatively poor colonization by *Escherichia coli* O157:H7 of the substrata, along with lack of persistence in the reactors, was not entirely expected. As noted, *E. coli* has been demonstrated as able to colonize pre-existing biofilms under relevant conditions (Swewzyk et al 1994, Robinson et al 1995). There are many possible reasons why this did not seem to occur at levels seen with the other test organisms in these experiments. This particular organism may preferentially remain in the bulk phase, this could agree with earlier speculation of the organism being diluted and flushed through the system by routine water usage in the Cabool, MO outbreak reports (Geldreich et al, 1992; Swerdlow et al, 1993). In our case the organism in the reactor would have to attach to a relatively smooth and uncolonized surface, and it may not act particularly well as a primary colonizer.

The observed results do not show that this organism will or will not colonize a biofilm, or persist in a drinking water distribution system. They merely show that, under the experimental conditions used, this organism did not readily colonize a relatively smooth and clean substratum, and was undetectable in the reactor bulk phase within 3 days of introduction of heterotrophs and a two hour reactor residence time. The low level of attachment compared to the other organisms tested is of interest, however. Also of consideration is that the strain used here was a clinical isolate and thus potentially not conditioned to water conditions, whereas all the other organisms tested were environmental isolates. All organisms were conditioned prior to inoculation to the reactors by leaving in a shake flask under low nutrient conditions as described in the Methods

section, but the physiological effects of this treatment were not examined.

Aeromonas hydrophila

The *Aeromonas hydrophila* data appeared to be variable between the experimental runs. Of the three runs performed, in two the test organisms rapidly became nonculturable and showed persistence as single cells in the biofilm. In the other, they went through a period of growth or resuscitation until chlorination was begun. Given the demonstrated potential for aeromonad growth in drinking water (Rippey et al, 1979; Van der Kooij, 1988; Havelaar et al, 1990), it would seem the latter case would be more representative of actual systems. In this experimental run, colony counts in the bulk phase diminished rapidly after small amounts of chlorine were added; this too seems representative of aeromonads in drinking water (unpublished data).

Klebsiella pneumoniae

Klebsiella is well known as an organism capable of growing in distribution systems (Geldreich and Rice, 1987). It is also well known as a biofilm-forming organism; several studies using the same strain as these experiments have utilized this quality (Huang et al, 1995; Stewart et al, 1994 and 1996). In the current experiments, it did appear to be the longest persisting organism of those tested, as well as the most chlorine resistant. However, it did not appear to be growing and increasing in numbers through the experiments, which was somewhat unexpected.

The extracellular polysaccharide formation capacity of this organism could be expected to provide some protection against disinfection. Total cell numbers were affected less from chlorination than organisms such as *Salmonella*. In one experimental run, *Klebsiella* remained culturable from the biofilm until the end of the experiment in spite of chlorination. This would agree with data from some distribution system blooms of coliforms remaining culturable in spite of heavy chlorination (Earnhardt, 1980; Lowther and Moser, 1984; van der Wende et al, 1989), of which *Klebsiella* is typical.

Utility of detection and identification methods

Selective media

Few difficulties were encountered using selective media in this study, it being a simple and well-established methodology. It is known to be accurate and sensitive, although this is dependant on the culturability of the organism as determined by its physiological condition. As demonstrated in this study, changes in this condition can make selective media increasingly unsuitable over time for detection and enumeration purposes.

With runs involving *Klebsiella* and *Aeromonas*, two types of selective media were used. Little difference was observed in the colony counts of the target organism between m-*Aeromonas* Agar and Ampicillin Dextrin Agar (ADA) for *Aeromonas*, and m-T7 Agar and m-*Klebsiella* Agar for *Klebsiella*. The m-T7 and ADA appeared to be less selective than their counterparts, invariably allowing the growth of more of the background population. This background growth was always able to be visually differentiated from the

target organisms on the basis of colony morphology.

Immunofluorescence

The use of this method produced the bulk of the data discussed in this report. The ability to detect nonculturable cells is the main advantage of this method, as well as the ability to examine intact biofilm samples for behaviour of target cells.

Numerous difficulties were encountered with this technique. The relatively low sensitivity of the method remained a problem throughout the experiments. This could have been compensated for by increasing the number of image fields examined, but the requirement for merging images made time the limiting factor in examining samples. Nonspecific staining was periodically a problem, particularly with the polyclonal antibody for *Aeromonas*, and required careful use of positive and negative controls. Overall, the staining procedure was generally reliable, although some samples were lost due to inadequate staining.

Determination of viability of persisting cells would be the most appropriate next step in microscopic examination of model systems such as the one used in this study. Combining CTC staining for viability determination with immunofluorescence for identification of cells has been demonstrated by Pyle et al (1995).

Another consideration is the range of antibodies commercially available. Antibodies against organisms of uncertain medical importance, such as *Klebsiella* and *Aeromonas*, are generally not commercially available, and must be specifically manufactured by researchers. Some potentially useful antibodies in common usage in

foreign countries, such as against the enterobacterial common antigen (manufactured by Riedel de Haen, Germany), can be difficult to obtain in this country due to the requirements of customs importation procedures.

PCR confirmation

Confirmation of colonies was quickly and reliably performed by this method where necessary. Given the increasing range of available primers and advances in cycling protocols, extension of this method to identification of other organisms has much potential as a standard laboratory technique.

Attempts in this study to extend this procedure to direct detection of target organisms from reactor samples were unsuccessful (unpublished data), primarily due to lack of sensitivity resulting from sample concentration procedures. These procedures are not discussed in this report.

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APPENDIX: DATA FROM EXPERIMENTAL RUNS

Appendix figure legend

The figures here show the enumeration of either the noted target organism or heterotrophs, in either the bulk phase or biofilm, over time.

Vertical dotted lines indicate a change in chlorine input to the reactor. The number to the right of the line indicates the concentration in mg L^{-1} of this input.

Horizontal dotted lines indicate that data was below detection level at this point in time. The point noted graphically is half of the minimum detectable value for that sample. For example, a count of $<1 \text{ CFU mL}^{-1}$ is represented as 0.5 CFU mL^{-1} .

The following abbreviations apply through this appendix.

XLD: Xylose Lysine Desoxycholate agar

2day: 2 day incubation on R2A agar at 35°C

7day: 7 day incubation on R2A agar at room temperature

Fab: Fluorescent antibody

PI: Propidium iodide

Rh123: Rhodamine 123

mT7: Modified tergitol-7 agar

mAA: m-Aeromonas Agar

ADA: Ampicillin Dextrin agar

mKA: m-Klebsiella agar

Figure 50: Sal Run 1 (Bulk Phase/Salmonella)

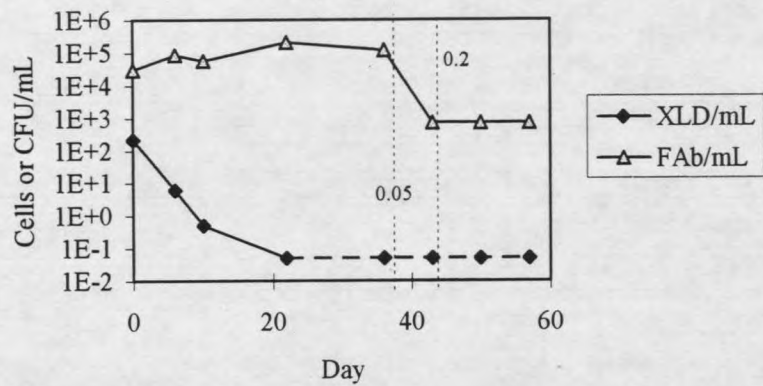


Figure 51: Sal Run 1 (Biofilm/Salmonella)

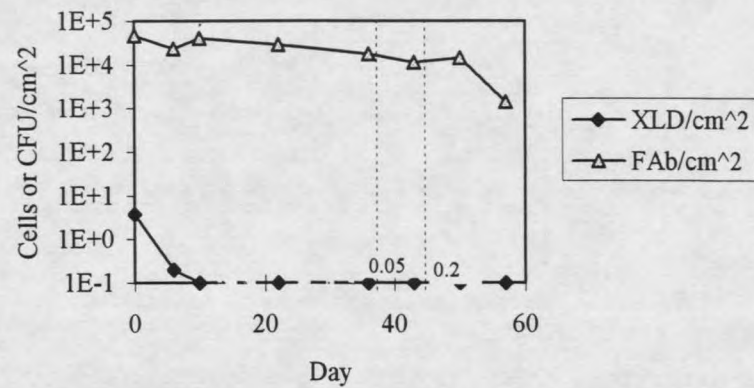


Figure 52: Sal Run 1 (Bulk Phase/Heterotrophs)

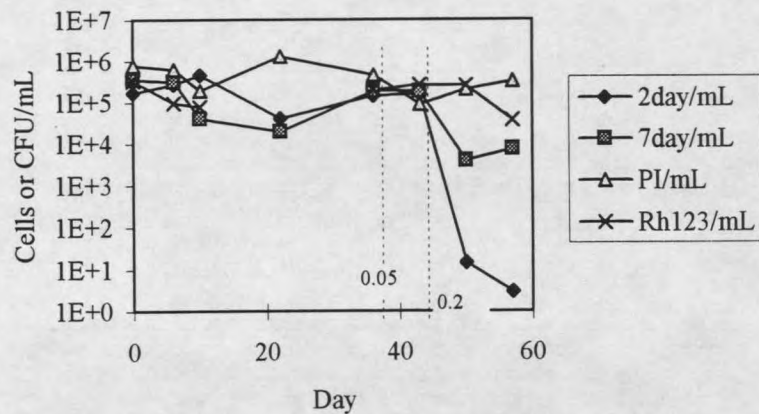


Figure 53: Sal Run 1 (Biofilm/Heterotrophs)

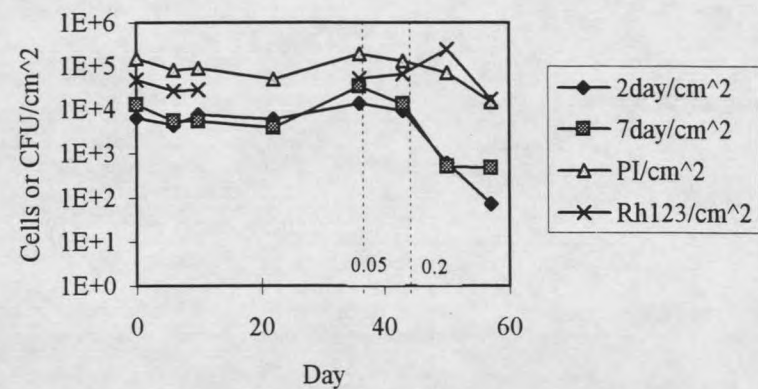


Figure 54: Sal Run 1C (Bulk Phase/Salmonella)

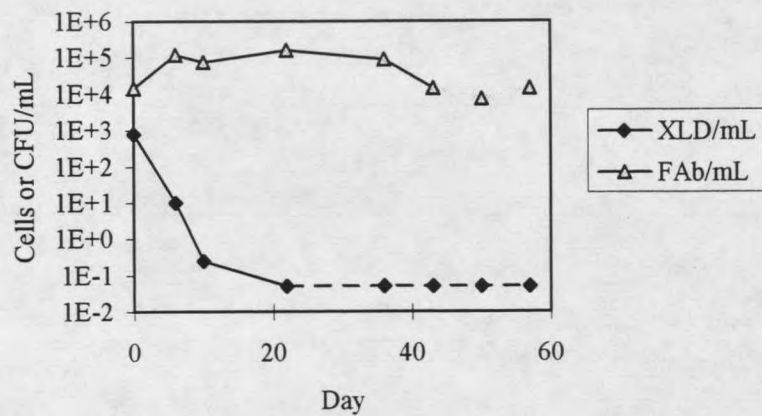


Figure 55: Sal Run 1C (Biofilm/Salmonella)

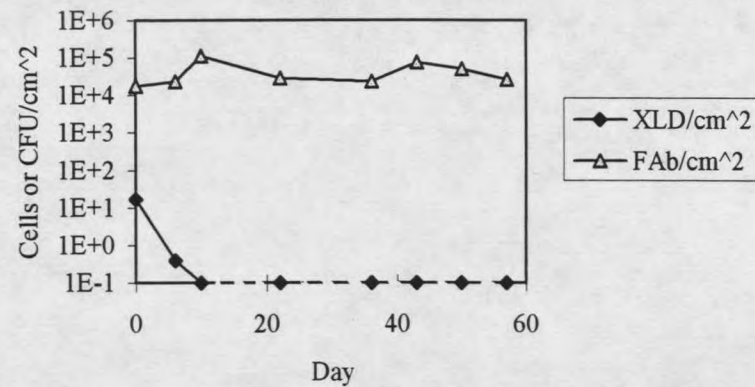


Figure 56: Sal Run 1C (Bulk Phase/Heterotroph)

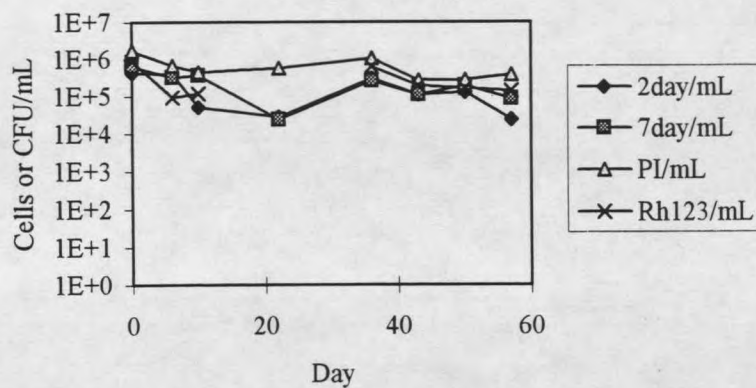


Figure 57: Sal Run 1C (Biofilm/Heterotrophs)

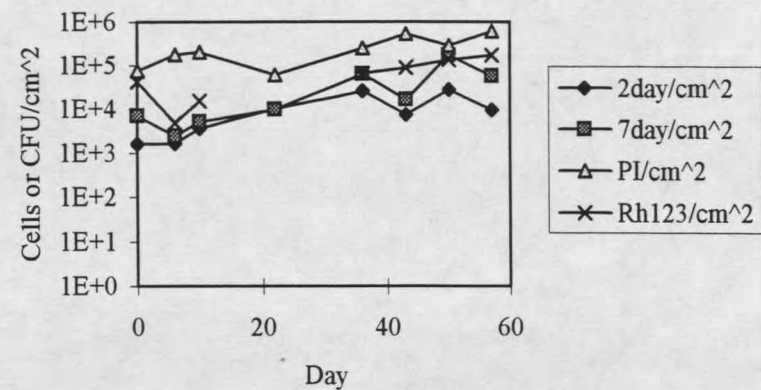


Figure 58: Sal Run 2 (Bulk Phase/Salmonella)

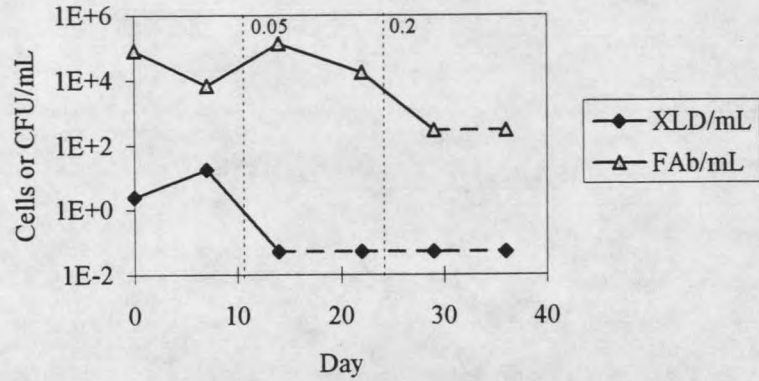


Figure 59: Sal Run 2 (Biofilm/Salmonella)

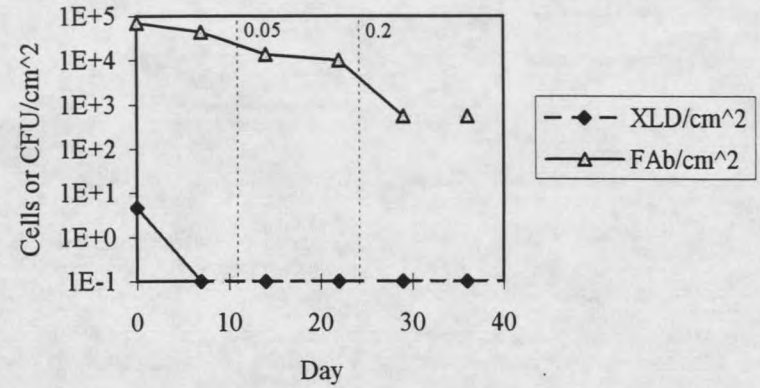


Figure 60: Sal Run 2 (Bulk Phase/Heterotrophs)

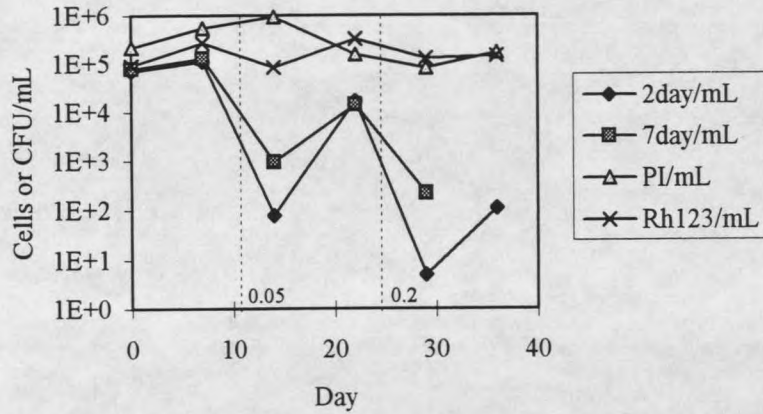


Figure 61: Sal Run 2 (Biofilm/Heterotrophs)

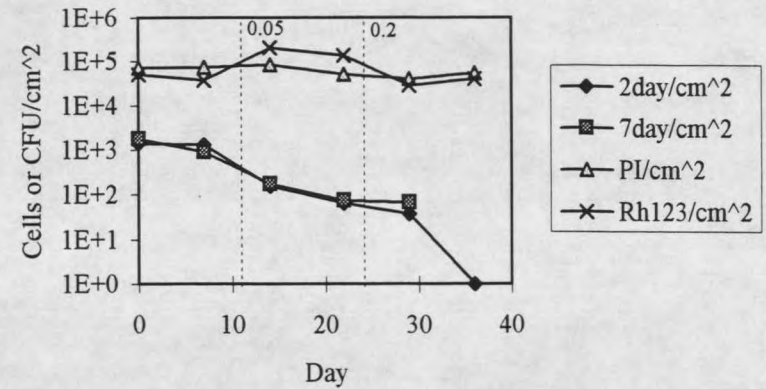


Figure 62: Sal Run 2C (Bulk Phase/Salmonella)

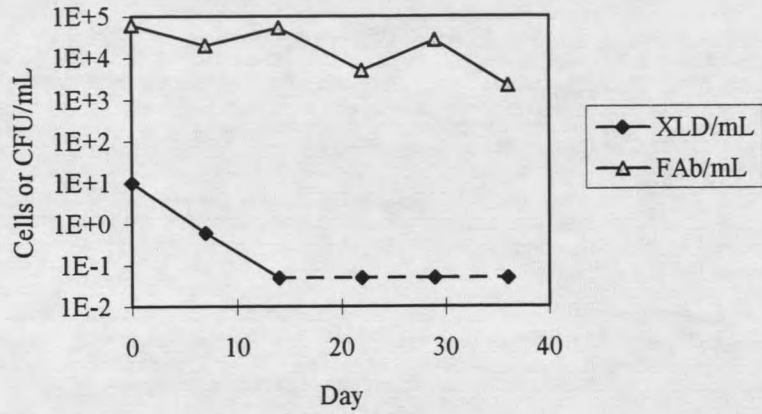


Figure 63: Sal Run 2C (Biofilm/Salmonella)

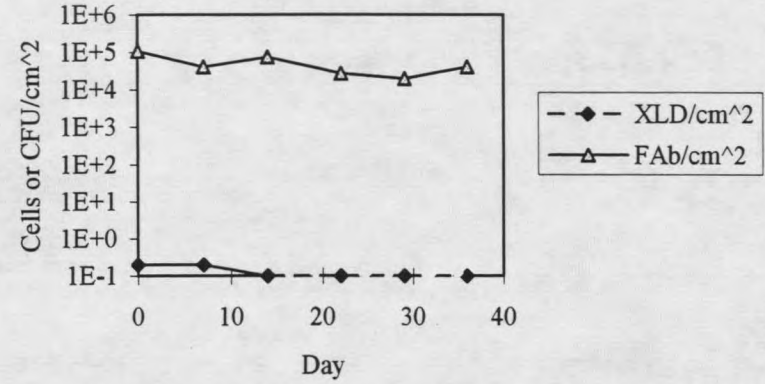


Figure 64: Sal Run 2C (Bulk Phase/Heterotroph)

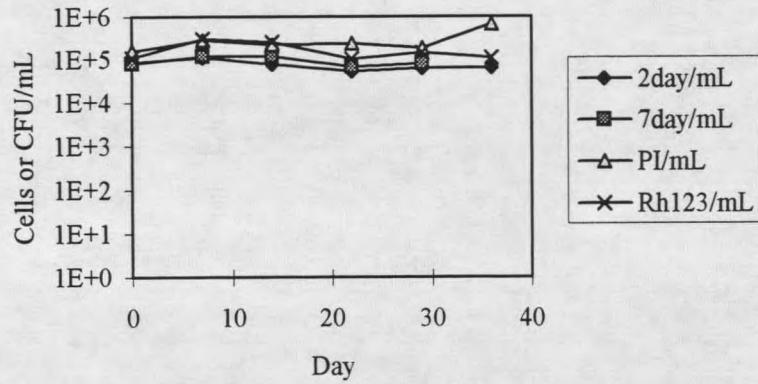


Figure 65: Sal Run 2C (Biofilm/Heterotrophs)

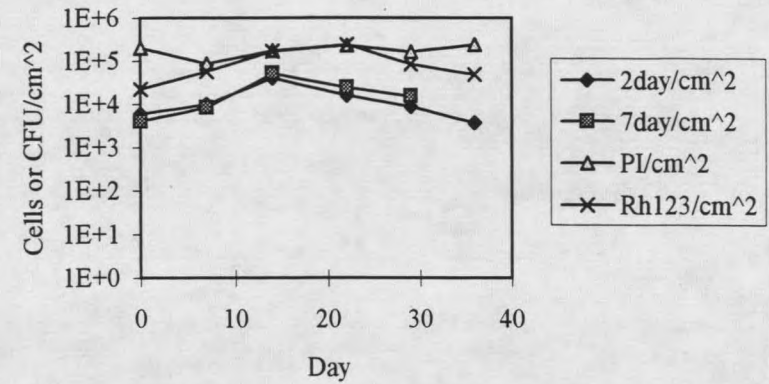


Figure 66: Sal Run 3 (Bulk Phase/Salmonella)

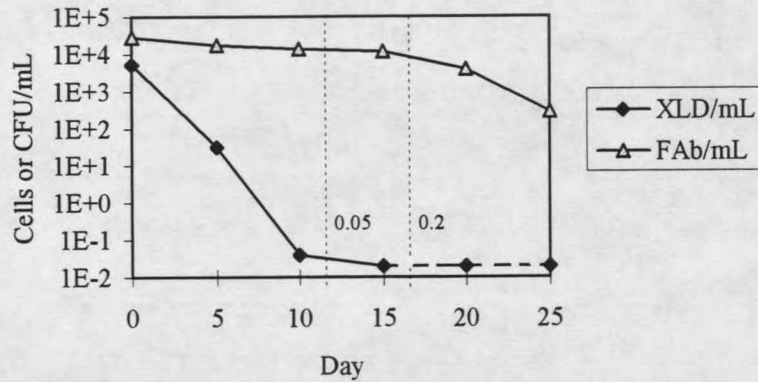


Figure 67: Sal Run 3 (Biofilm/Salmonella)

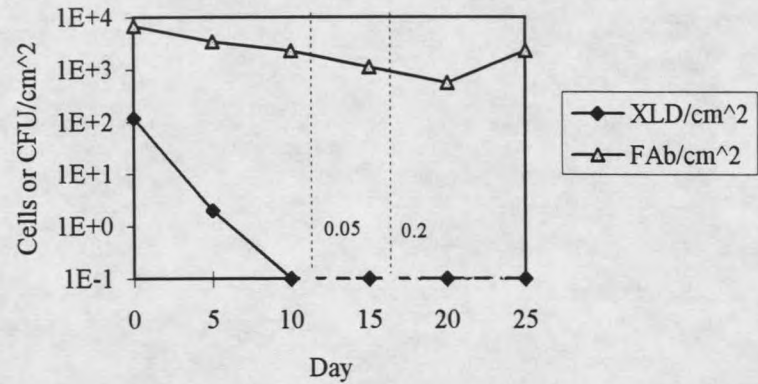


Figure 68: Sal Run 3 (Bulk Phase/Heterotrophs)

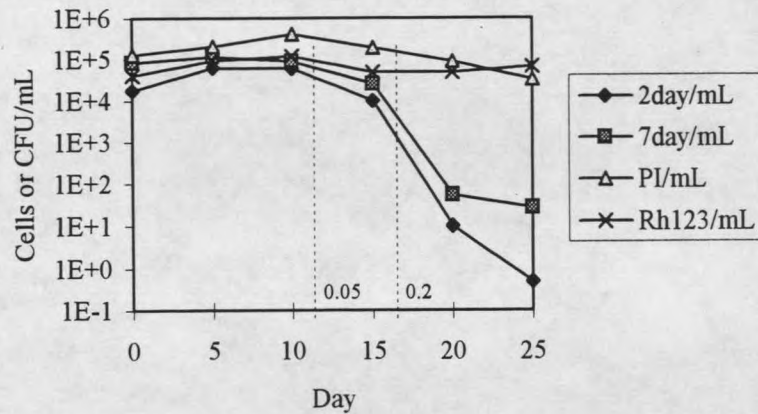


Figure 69: Sal Run 3 (Biofilm/Heterotrophs)

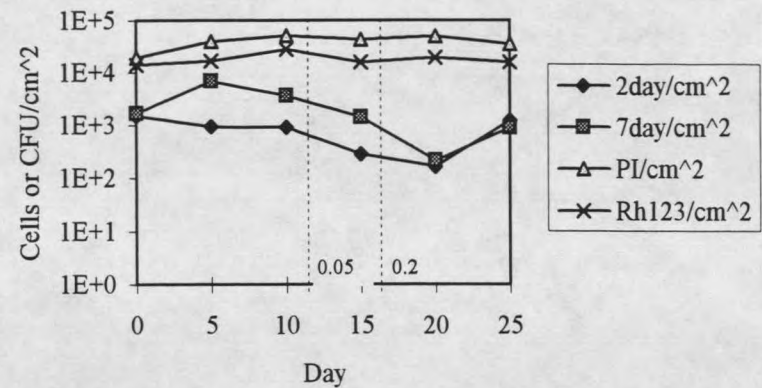


Figure 70: Eco Run 1 (Bulk Phase/Ecoli)

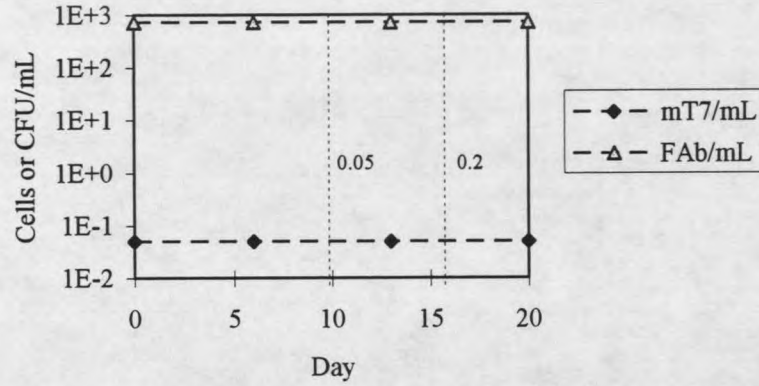


Figure 71: Eco Run 1 (Biofilm/Ecoli)

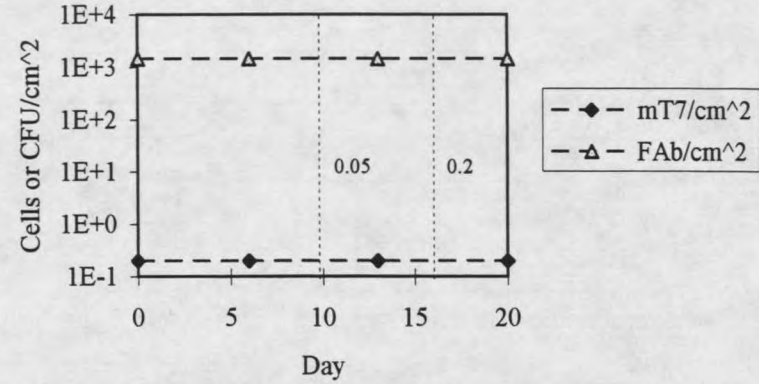


Figure 72: Eco Run 1 (Bulk Phase/Heterotrophs)

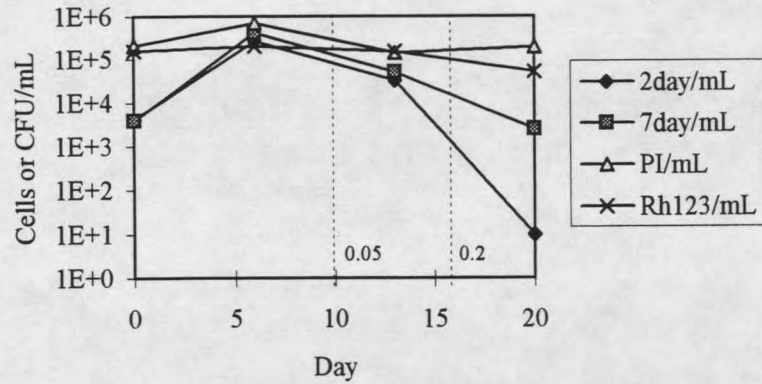


Figure 73: Eco Run 1 (Biofilm/Heterotrophs)

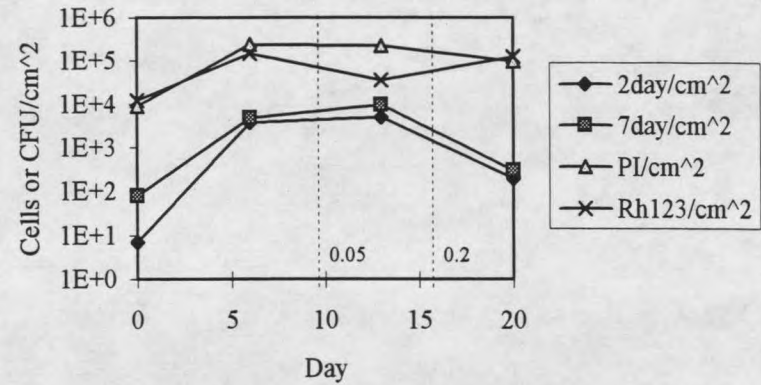


Figure 74: Eco Run 1C (Bulk phase/Ecoli)

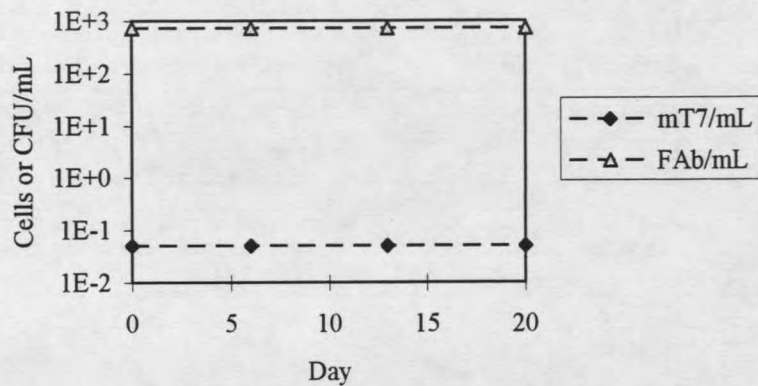


Figure 75: Eco Run 1C (Biofilm/Ecoli)

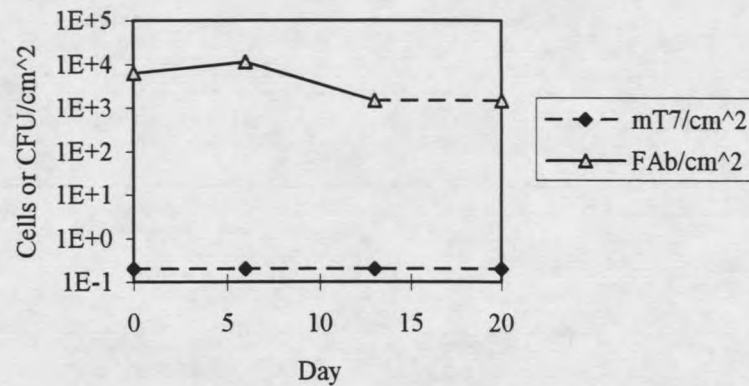


Figure 76: Eco Run 1C (Bulk Phase/heterotroph)

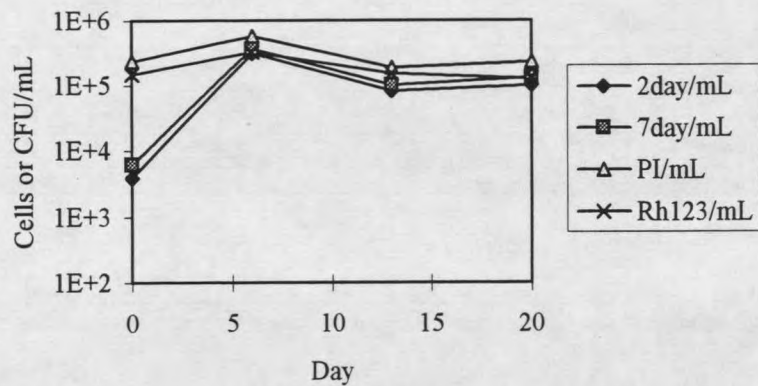


Figure 77: Eco Run 1C (Biofilm/Heterotrophs)

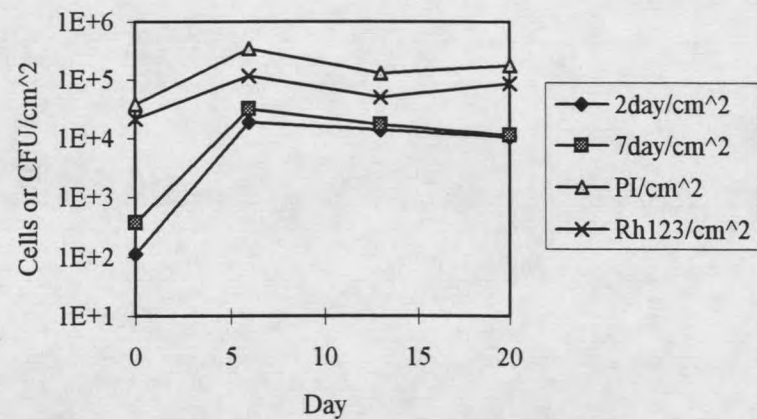


Figure 78: Eco Run 2 (Bulk Phase/Ecoli)

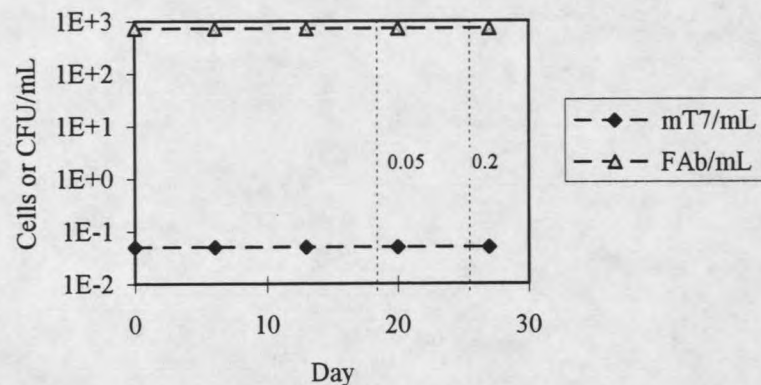


Figure 79: Eco Run 2 (Biofilm/Ecoli)

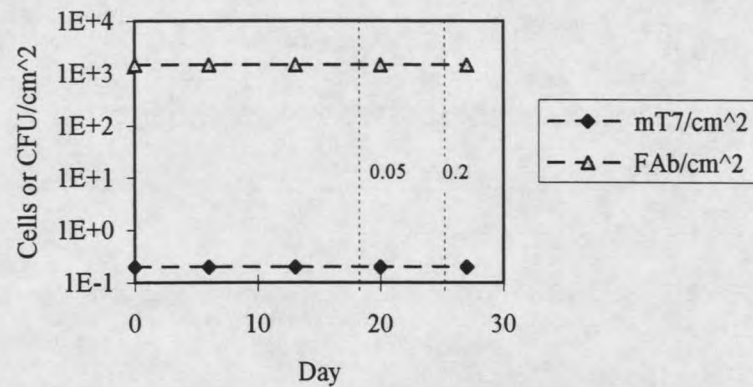


Figure 80: Eco Run 2 (Bulk Phase/Heterotrophs)

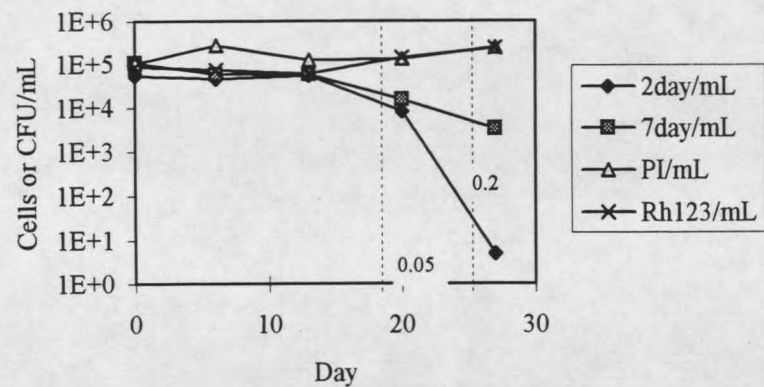


Figure 81: Eco Run 2 (Biofilm/Heterotrophs)

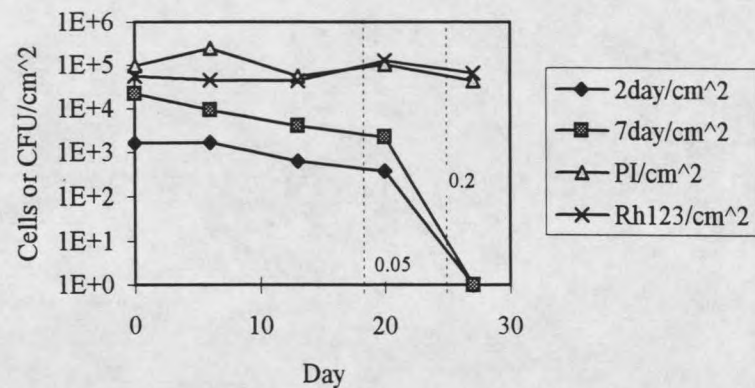


Figure 82: Eco Run 2C (Bulk Phase/Ecoli)

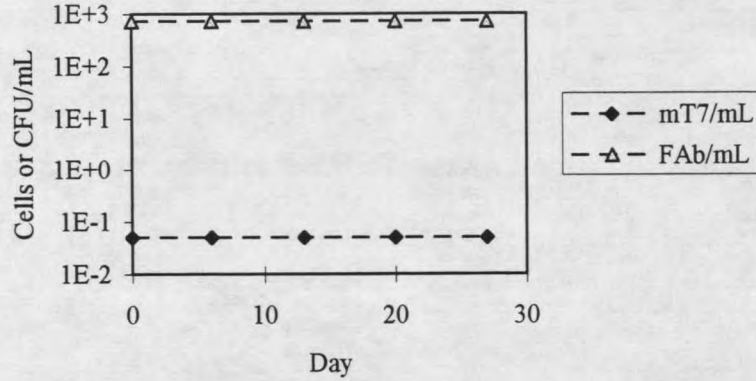


Figure 83: Eco Run 2C (Biofilm/Ecoli)

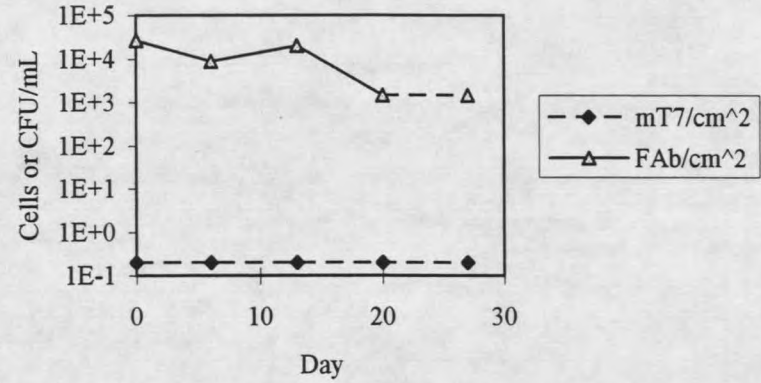


Figure 84: Eco Run 2C (Bulk Phase/heterotroph)

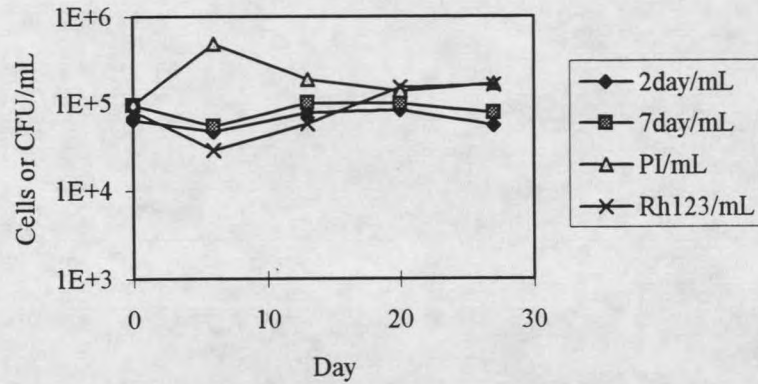


Figure 85: Eco Run 2C (Biofilm/Heterotrophs)

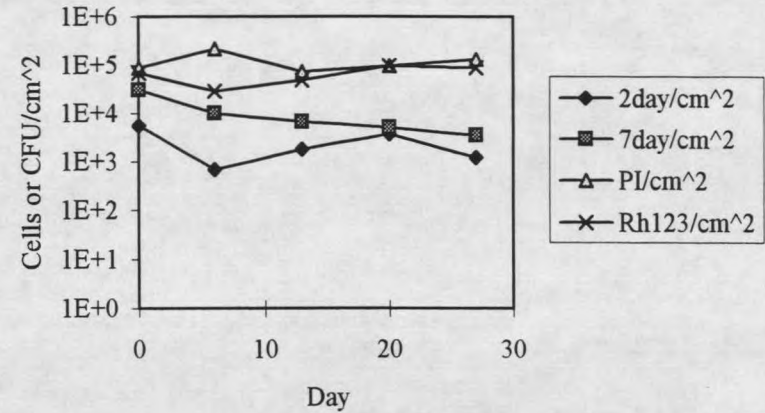


Figure 86: Aero Run 1 (Bulk phase/Aeromonas)

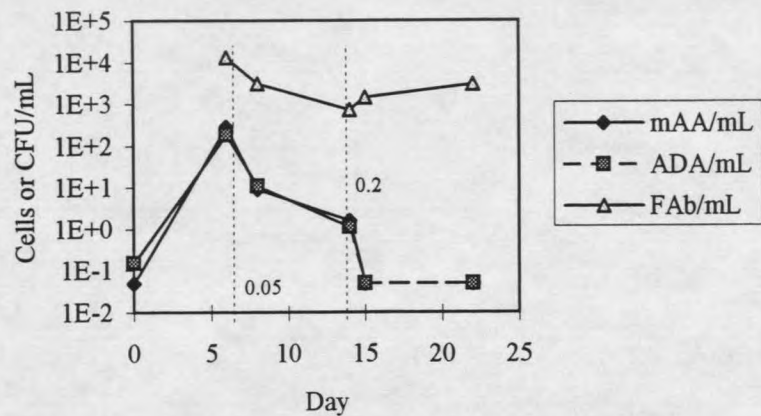


Figure 87: Aero Run 1 (Biofilm/Aeromonas)

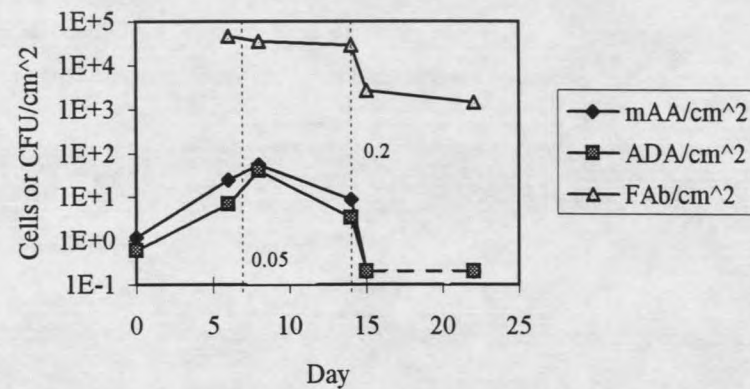


Figure 88: Aero Run 1 (Bulk phase/Heterotroph)

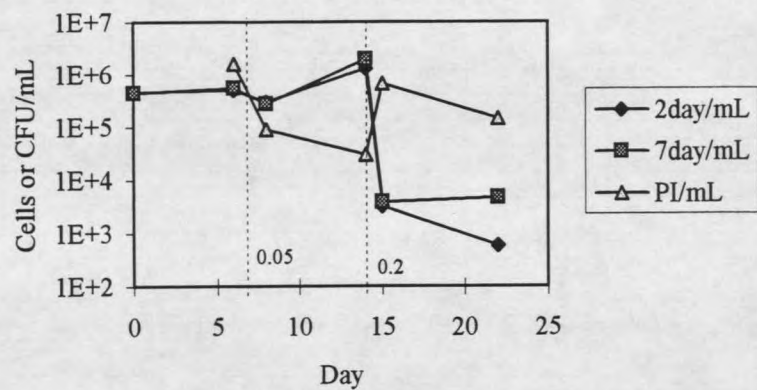


Figure 89: Aero Run 1 (Biofilm/Heterotrophs)

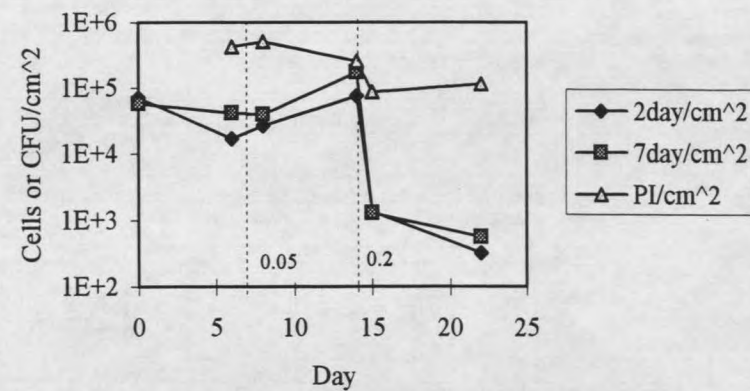


Figure 90: Aero Run 2 (Bulk Phase/Aeromonas)

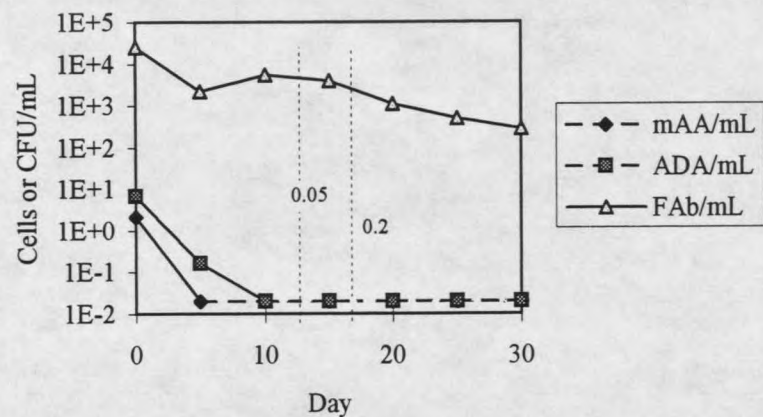


Figure 91: Aero Run 2 (Biofilm/Aeromonas)

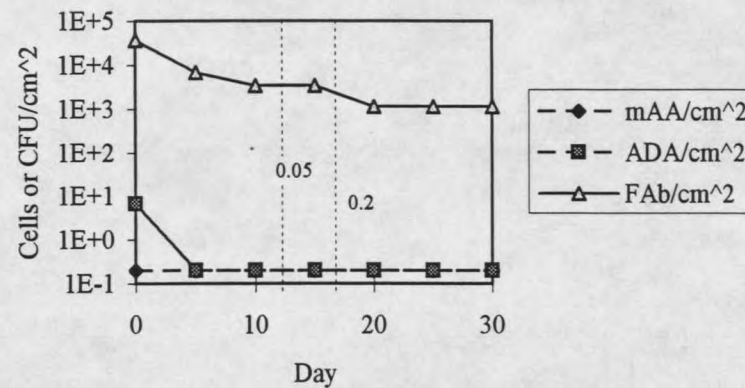


Figure 92: Aero Run 2 (Bulk Phase/Heterotroph)

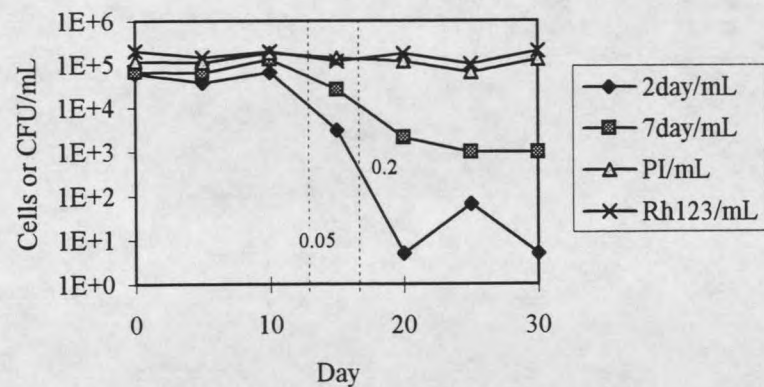


Figure 93: Aero Run 2 (Biofilm/Heterotrophs)

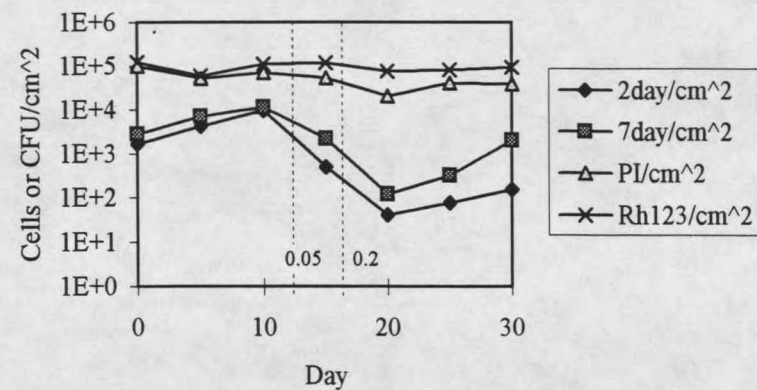


Figure 94: Aero Run 3 (Bulk Phase/Aeromonas)

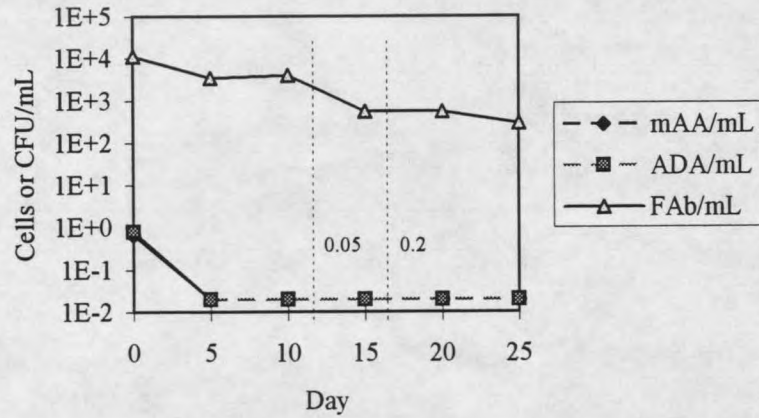


Figure 95: Aero Run 3 (Biofilm/Aeromonas)

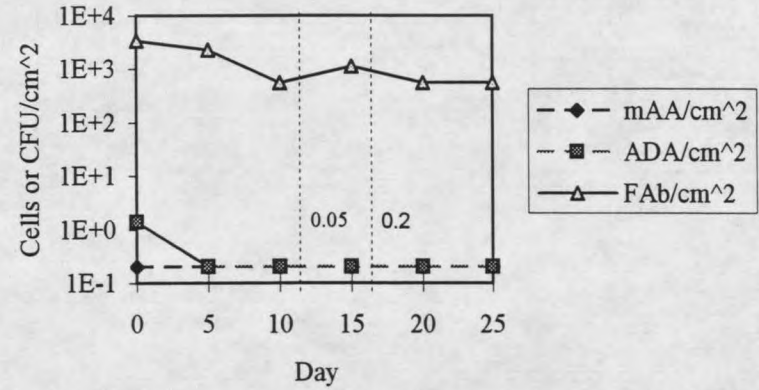


Figure 96: Aero Run 3 (Bulk phase/Heterotroph)

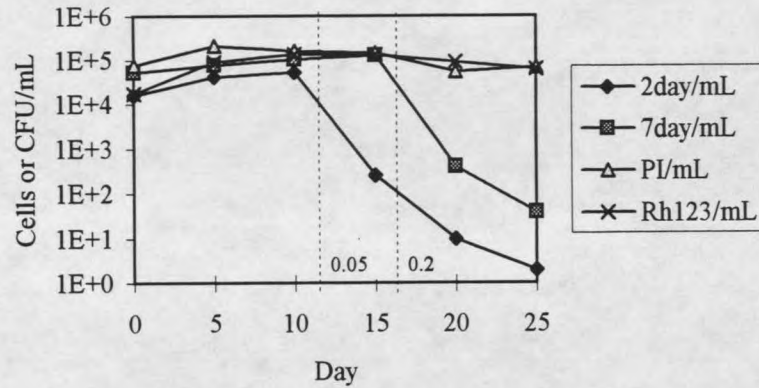


Figure 97: Aero Run 3 (Biofilm/Heterotrophs)

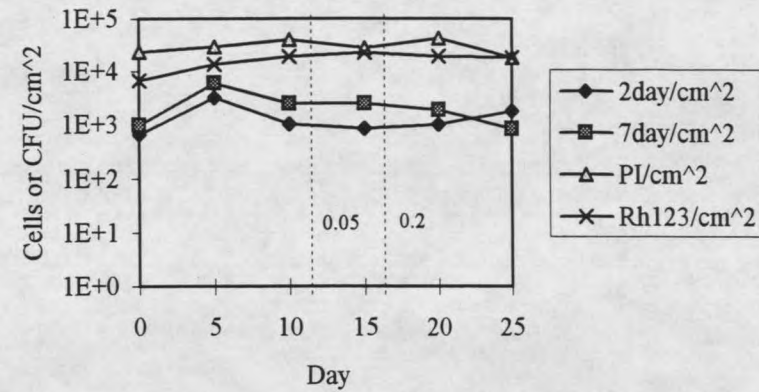


Figure 98: Kleb Run 1 (Bulk Phase/Klebsiella)

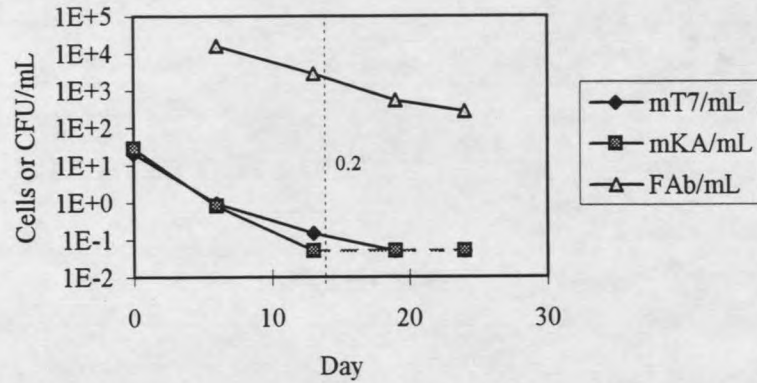


Figure 99: Kleb Run 1 (Biofilm/Klebsiella)

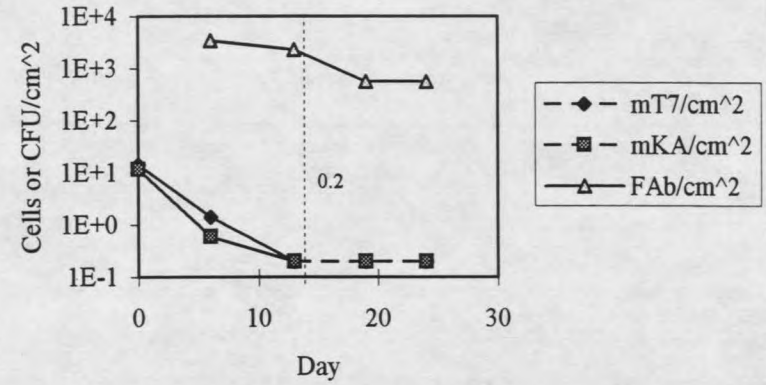


Figure 100: Kleb Run 1 (Bulk Phase/Heterotrophs)

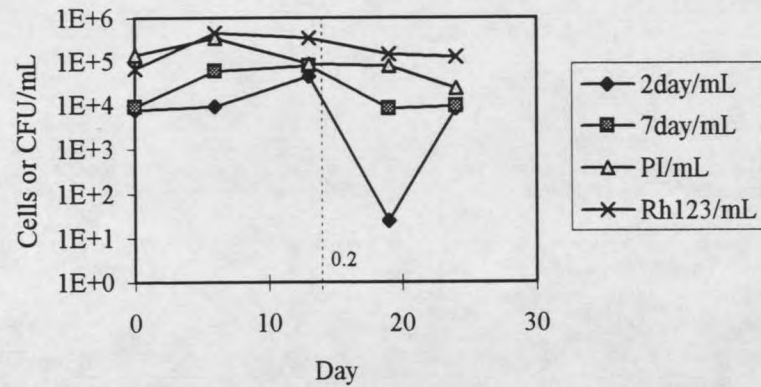
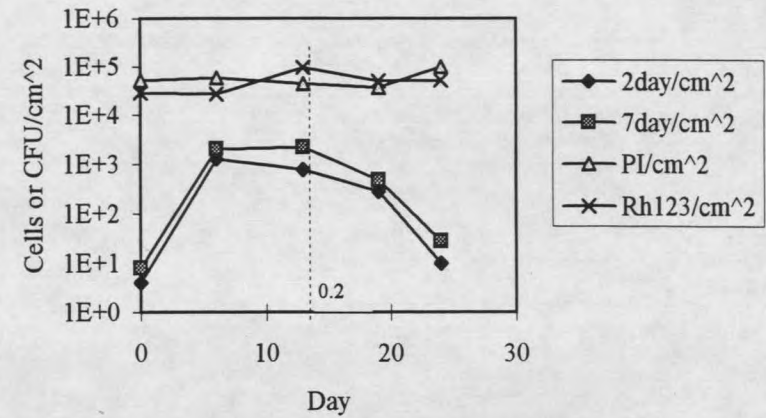


Figure 101: Kleb Run 1 (Biofilm/Heterotrophs)



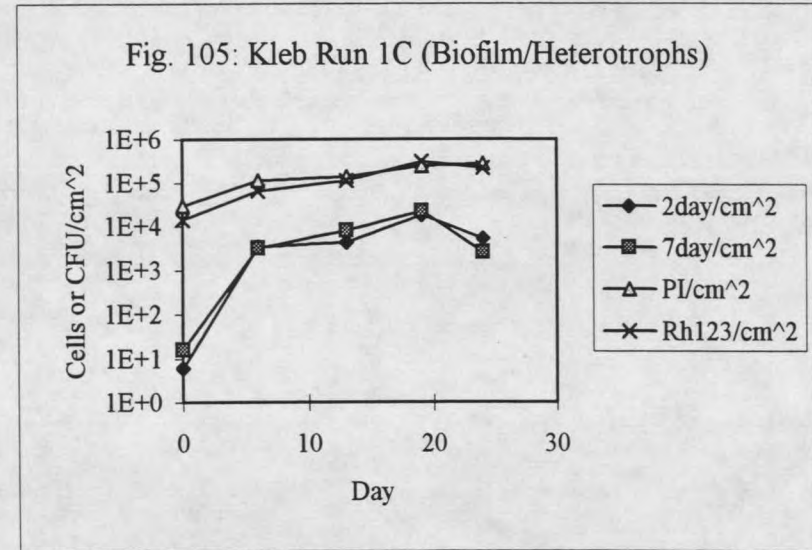
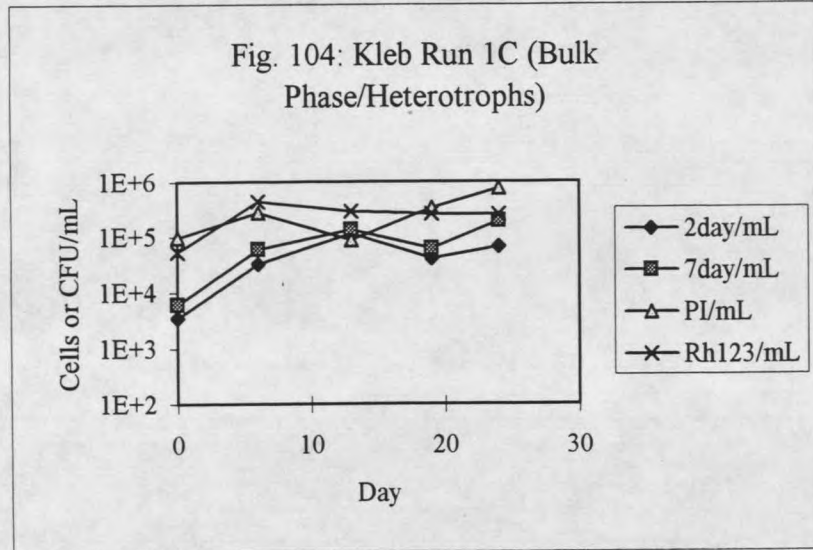
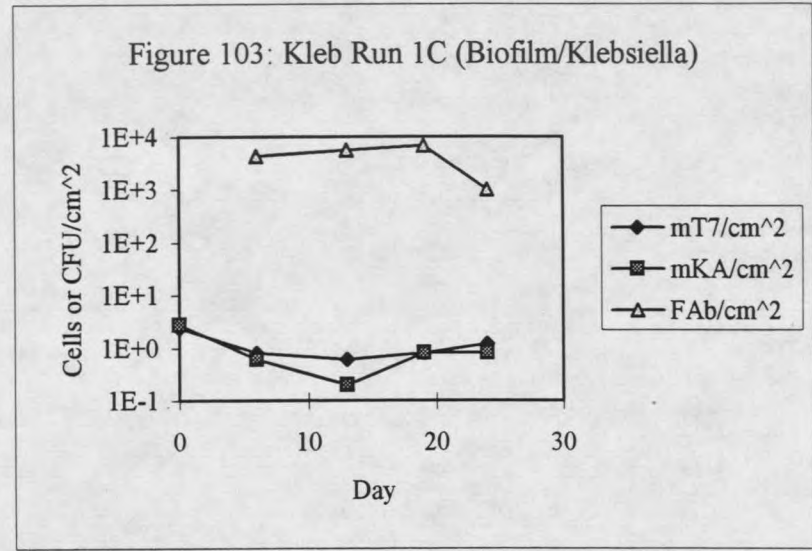
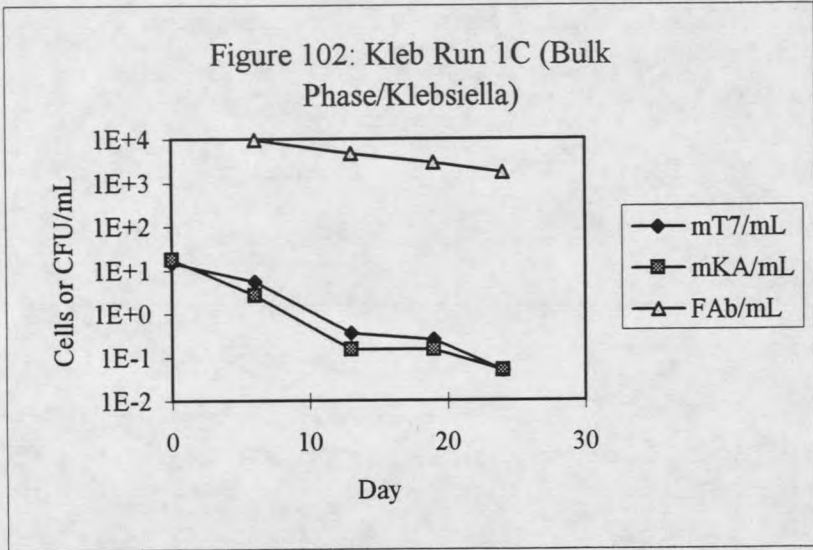


Fig. 106: Kleb Run 2 (Bulk Phase/Klebsiella)

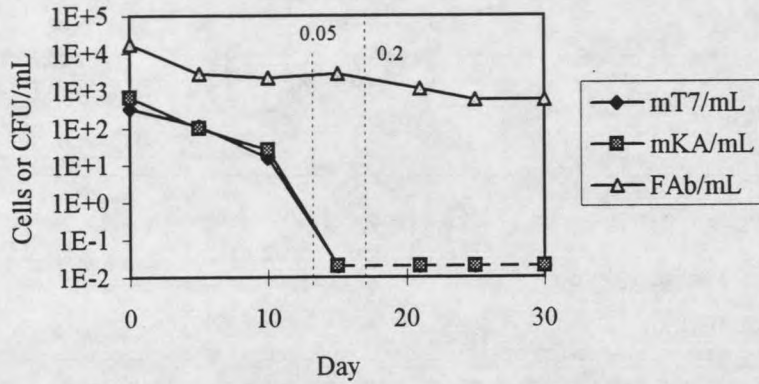


Fig. 107: Kleb Run 2 (Biofilm/Klebsiella)

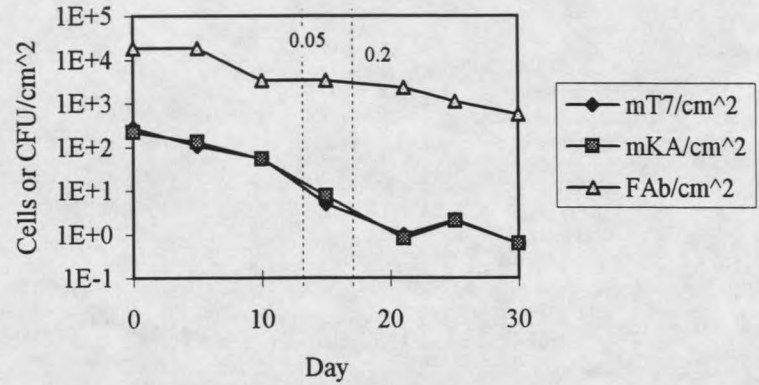


Fig. 108: Kleb Run 2 (Bulk Phase/Heterotrophs)

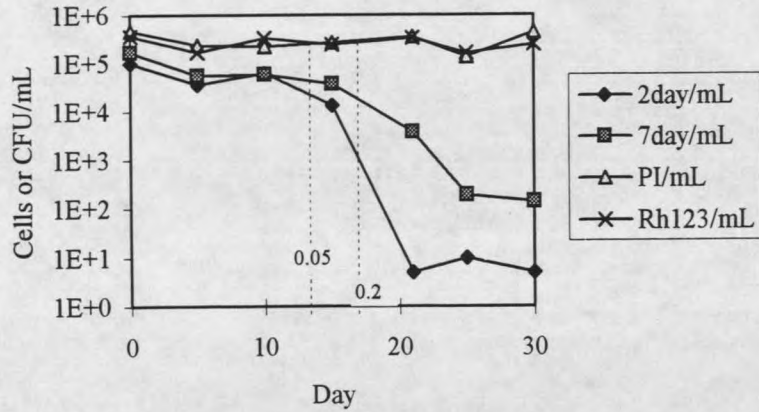
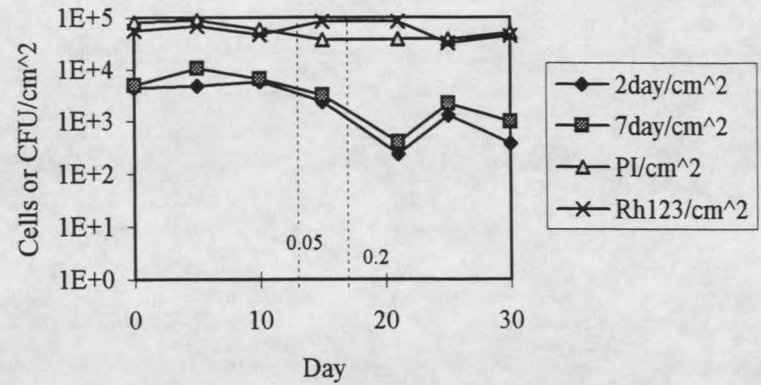


Fig. 109: Kleb Run 2 (Biofilm/Heterotrophs)



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